PRE-HARVEST SPROUTING IN WHEAT

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DECLARATION

This thesis was composed by myself and describes my own original work. It has not been submitted for a degree at any other University.

CONTENTS

ABSTRACT

This work examines some of the factors that influence PHS in wheat, with particular emphasis on the premature production of alpha-amylase (PMAA) in the absence of visible sprouting.

Field trials in 1989 and 1990 studied alpha-amylase activity in relation to grain development in four winter wheat cultivars. Weather conditions were generally warmer and drier than average in both years. Differences in drying rate were induced by covering and wetting treatments. Fenman had a slower rate of grain drying (40%-23% moisture) than other varieties, but, within varieties, drying rate appeared not to be related to alpha-amylase levels. This lack of relationship was possibly due to the fact that all of the drying rates were relatively fast, and, apart from Fenman, levels of alpha-amylase were low. The level of dormancy and the lack of visible sprouting indicated that the alpha-amylase activity observed in Fenman was due to PMAA. The increase in alpha-amylase began when moisture percentage was between 40 and 35 %. Activity rose steeply, and remained high until harvest. There were differences in alpha-amylase activity between the wetting and covering treatments and the control in 1989, but these were not related to effects on grain drying. There was an increase in the number of grains with high alpha-amylase activity in the covered and wetted treatment and it was suggested that changes in microclimate at an earlier stage of grain development may have affected sensitivity to GA, leading to subsequent development of PMAA. This was investigated in 1990. The onset of sensitivity to GA_3 coincided with the increase in alpha-amylase activity in Fenman, but GA_3 sensitivity was also apparent in other cultivars that did not exhibit PMAA. Fenman showed an earlier onset of GA-sensitivity than the other varieties, but neither covering nor wetting had a significant effect on GA-sensitivity or PMAA.

The possible role of GA in the regulation of PMAA was investigated in a trial comparing near-isogenic lines of Maris Huntsman, differing in GA-sensitivity. Levels of alpha-amylase were reduced in dwarf (*Rht1+2* and *Rht3*) and semi-dwarf (*Rht1* and *Rht2*) lines relative to the tall (*rht*) genotype. However, this did not appear to be related to variation in GA-sensitivity shown by developing grains. *Rht1* showed a greater response to GA_3 than the other lines. It was suggested that the genotypes may differ in sensitivity to environmental effects.

An attempt to identify critical stages of grain development, with respect

to environmental effects on PMAA, was unsuccessful, but did indicate that constant warm and dry environmental conditions reduce alpha-amylase activity in Fenman.

Ear culture was used to examine the effect of drying rate on PMAA in Maris Huntsman. There was no apparent relationship, possibly due to relatively fast drying rates, but it is suggested that ear culture is a suitable system in which to study environmental effects.

There is considerable variation in PMAA both within and between ears, and possible reasons for this were considered. Non-destructive methods of single grain moisture determination were used to examine the relationship between alpha-amylase activity and grain moisture content. Response to GA_3 appeared to vary between grains in the same ear, but there were too few data to relate this to incidence of PMAA. There appeared to be an asymmetric distribution of alpha-amylase within grains.

It is suggested that environmental factors may cause an earlier onset of GA-sensitivity, and that this may coincide with a moisture content sufficient to allow alpha-amylase synthesis to begin.

ABA	Abscisic acid.
ATP	Adenosine tri-phosphate.
BPNPG7	Blocked p-nitrophenyl maltoheptaoside.
CN	Cyanide.
DAA	Days after anthesis (actual)
"DAA"	Developmental days after anthesis.
DI	Dormancy index.
EDTA	Ethylenediaminetetra-acetic acid.
FAO	Food and Agriculture Organisation of the United Nations.
GA	Gibberellic acid.
HFN	Hagberg Falling Number.
H-GCA	Home-grown cereals authority.
pI	Isolectric point.
Met. Office	Meteorological office.
NADP	Nicotinamide adenine dinucleotide-phosphate.
NADPH	Nicotinamide adenine dinucleotide-phosphate (reduced form).
NMR	Nuclear magnetic resonance.
P	Probability.
PEG	Polyethylene glycol.
PHS	Pre-harvest sprouting.
PMAA	Pre-maturity alpha-amylase.
RH	Relative humidity.
mRNA	Messenger ribonucleic acid.
SE	Standard error.

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1. Introduction and literature review.

1.1 Production and usage of wheat.

The cereals are a group of agriculturally important grasses within the Gramineae family, grown primarily for their seeds. They are the most important plant group in world agriculture, forming the staple diet of the majority of the world's population. They are also important as animal feeds, or may be processed for industrial uses. Within the cereals, wheat is the major crop, both in terms of production area and total production (Table 1.1).

	Area harvested (hectares x 10^{-6})	Production (tonnes x 10^{-6})
Wheat	225.8	541.77
Rice	148.39	517.57
Maize	129.3	470.65
Barley	72.74	167.9
Sorghum	44.7	59.91
Oats	22.88	41.62
Rye	16.91	36.11
Millet	37.41	29.96

Table 1.1. World production of cereal crops, 1989 (FAO,1991)

Wheat is adaptable to a range of environmental conditions and is the most widely cultivated food crop. It is best suited, however, to temperate regions, and the bulk of world wheat production is in the northern hemisphere (Table 1.2).

Wheat is processed in a variety of ways for human food. It may be milled and used to produce baked products eg. bread, biscuits, pastry, and cakes, or flaked, puffed, rolled or shredded for use in breakfast cereals. Pasta is usually made from durum wheat (*Triticum durum*) but noodles are made from white wheat. Wheat flour and wheat starch may be used as thickeners in soups etc. Gluten may be extracted and used to produce high-protein flours for bread-making. Wheat may also be used as a cereal source in grain whisky distilling, In addition to its use as human food, wheat is also grown for animal feed use and as a seed crop. In addition, wheat starch has many industrial uses.

	Area harvested (hectares x 10^{-6})	Production (tonnes x 10^{-6})
USSR	47.68	92.3
China	29.84	90.81
USA	25.17	55.43
India	24.11	54.11
France	5.01	31.82
Canada	13.63	24.58
Turkey	9.23	16.22
Germany	2.55	14.51
Pakistan	7.73	14.42
Australia	8.94	14.12
UK	2.08	14.03
Argentina	5.35	10.1
Poland	2.2	8.46
Romania	2.32	7.88
Italy	2.9	7.41

Table 1.2. Major wheat producing countries, 1989 (FAO,1991)

1.2 Pre-Harvest Sprouting.

Any factor that reduces yield and/or quality of wheat is of significant economic importance. In many of the wheat-growing regions of the world pre-harvest sprouting (PHS) is a problem. In simple terms, PHS is the germination of a grain while still attached to the parent plant prior to harvest. It is usually a result of rain falling on after-ripened grains from which dormancy has been lost, but, less commonly, may also occur prior to the onset of dormancy. PHS has many adverse effects, on both yield and quality. In particular, breadmaking quality is badly affected, mainly due to the excessive levels of alpha-amylase and other germination enzymes produced during sprouting. The financial cost to growers and millers and, ultimately, consumers, has meant that considerable research interest has long been directed at the problem of PHS (eg. Hutchinson *et al.*, 1948).

The area traditionally most affected by PHS is the moist temperate region of Scandinavia and northwest Europe (Meredith and Pomeranz, 1985), but it is a potential problem in any climatic region where cool and/or wet conditions prevail prior to, and during harvest. Thus, the northern wheat belt of

Australia, much of central South America, the Pacific Northwest of America, the Ontario province of Canada, large areas of India and Pakistan, and parts of East and Southern Africa are regularly affected. The most-recent worldwide economic effects of PHS have been calculated by Derera (1990) and the costs to some of the major wheat producing countries are summarised in Table 1.3.

Country	Incidence of PHS (no. years), 1978-1988	Estimated average loss/year (US$ M)
Canada	4	101.4
USA	5	NA
UK	3	30
Germany	3	16+ *
Poland	3 (1984-1988)	92
Australia	3 (1984-1988)	19

* does not include cost to former GDR.

Table 1.3. Incidence of PHS and estimated economic losses in major wheat producing countries.

PHS may be a problem in other cereal crops, but does not usually involve such heavy financial losses. This reflects the fact that the most damaging effect of PHS is on breadmaking quality, and other cereals, apart from rye, are not used for breadmaking. A less well known aspect of PHS is the phenomenon of prematurity alpha-amylase (PMAA), in which alpha-amylase is produced by pre-ripe grains in the absence of visible sprouting, with equally damaging effects on breadmaking quality. Although the propensity of some UK wheat varieties to contain high levels of alpha-amylase in ungerminated grain had been recognised (Bingham and Whitmore, 1966), and the potential damaging effects discussed (Gale *et al.*, 1983), there are no reports of widespread damage due to PMAA until 1985, an exceptionally wet and cool harvest year, when low UK Hagberg Falling Numbers (HFN) were attributed to PMAA (Gale and Lenton, 1987; Flintham and Gale, 1988). PMAA has also been described in South Africa, in "perfect" harvesting conditions (Marais and Kruis, 1983) and in Australia, in the absence of rain (Mares, 1987a). Sprouting damage is commonly measured in harvested grain by the HFN method gives an indirect estimate of alpha-amylase activity. It is difficult to determine the relative importance of PMAA to sprouting damage as a whole. Thus it is likely

that PMAA has been responsible for low HFNs in other wheat-producing countries but has not been identified as such. The most recent occurrences of "sprouting" (both PHS and PMAA) in the UK are outlined in Table 1.4 (from Derera, 1990).

Year	Production (tonnes x 10^{-6})	Value US $ M	% production < 180 HFN	Loss US $ M
1977	4.7	643.7	77	25.1
1985	15	2925	61	108.9
1987	13.9	2700	61	113.8

Table 1.4 Incidence of sprouting in the UK winter wheat crop.

1.3 Sprouting Damage.

Some of the damaging effects of PHS are common to all cereal crops. For instance, yield will be reduced when sprouted and shrivelled grains are lost along with the chaff and straw during combine harvesting (Belderok, 1968). In addition, dry matter losses due to breakdown of storage carbohydrates will reduce the weight of those grains that are harvested. Quality of seed crops may be damaged by PHS. Fully-sprouted grains are obviously not viable, but germination percentage can be reduced even before visible sprouting occurs. In addition, loss of viability during storage is more rapid in affected seed lots (Agrawal and Dadlani, 1984; Elias and Copeland, 1989). However, it is the effect of excessive alpha-amylase on breadmaking quality of wheat that is the major economic effect of PHS (and PMAA), and therefore this will be considered in more detail.

During the breadmaking process, heating and addition of moisture cause starch gelatinisation and if alpha-amylase is present in excessive amounts hydrolysis of starch may begin. The initial hydrolysis is very rapid and is thought to involve random hydrolysis of α-(1-4)-bonds in amylose and amylopectin molecules, thereby producing many short-chain dextrins and leading to a large decrease in viscosity (Hill and MacGregor, 1988). These dextrins may make the dough softer and stickier, but in rapid bread-making methods (such as the widely-used Chorleywood process) the real effect is not apparent until after baking when it is attempted to slice the loaf. Affected loaves have a wet and sticky crumb texture that is impossible to slice cleanly (Meredith and Pomeranz, 1985). If soluble sugars, a later product of starch hydrolysis, are present, the

loaf may also be undesirably darkly coloured.

Similar amylolytic starch hydrolysis has damaging effects on the quality of other wheat flour products such as noodles, flat bread and chapattis (Orth and Moss, 1987). In starch and gluten extraction, alpha-amylase damage will lead to a higher percentage of unusable soluble compounds, and a reduced starch quality (Jones, 1987).

The HFN test gives an indirect measurement of alpha-amylase, based on the liquidity of a gelatinised flour and water mixture, and is used by millers as a routine quality test for bread-making wheat samples. HFN values below 220 are unacceptable. In 1985 and 1987 only 17.3% and 20.3% of the UK bread wheat crop achieved this standard (Stevens *et al.*, 1988), necessitating high cost imports of Canadian wheats.

1.4 Grain Development.

In order to understand the processes involved in the production of alpha-amylase in pre-harvested grains it is necessary to have an understanding of the structure, physiology and biochemistry of the developing wheat grain and these will be discussed in the next section, with emphasis on those factors thought to be involved in sprouting damage.

One of the problems encountered when reviewing the literature on grain development is the fact that environment will affect the timescale, such that an Australian grown crop at 20 days after anthesis will be more advanced than, say, a Scottish crop of the same chronological age. Attempts have been made to overcome this with descriptive development scales but there is no one scale in common usage. Although the Zadoks scale (Landes and Porter, 1989) is in widespread use to describe vegetative development, its use is rare during grain development. Riffkin (1987) developed an expanded descriptive scale to cover grain development from anthesis until the stage where grains were "hard and floury, becoming brittle". The main problem with this, and other descriptive scales however, is that little visible change occurs after this stage, so, although biochemical and physiological events are still ocurring, there is no morphological marker to use as an indication of timescale. Kirby and Appleyard (1986) note that grain water content is the criterion by which husbandry decisions are generally taken, but Gordon *et al.* (1979) have found that measured maturity traits do not consistently coincide with particular moisture contents, indicating that water content is not a sufficiently accurate measure to use as a marker of biochemical and physiological events in the grain.

In the absence of such a marker, terms such as "Harvest-Ripe" (HR) and "yellow-ripe" (YR) continue to be used. By implication, grains described as "Harvest-Ripe" are about to be detached from the parent plant, and such grains usually represent the terminal sample in studies of PHS and PMAA. However, as Gordon *et al.* (1979) discuss, if combine harvester moisture requirements are taken to define "harvest ripeness" the critical moisture concentration can range from 12-22% (wet basis). For their study, in Australia, a moisture concentration of 12% was taken to represent HR and they note that this would be 17.5% or 20% in cooler ripening conditions.

1.4.1 Floral structure and pollination.

A spikelet of wheat (Fig. 1.1a) may contain one or more florets. The wheat flower (Fig 1.1b) consists of a single-celled ovary, surmounted by two styles terminating in feathery stigmas, and three stamens with large anthers. Surrounding and protecting these structures are the lemma and palea. At anthesis, the lodicules at the base of the ovary swell, forcing apart the lemma and palea and revealing the stigma and anthers. Anther dehiscence precedes flower opening and this means that self pollination is normal. Fertilization occurs when a male nucleus from the pollen tube fuses with an egg nucleus of the ovule to form a zygote. At the same time, a second male nucleus fuses with two polar nuclei in the endosperm mother cell. This will develop into the endosperm. The ovary wall persists to form the pericarp, cross cell layer, and tube cells of the developing fruit, whilst the nucellar tissue and the inner integuments of the ovule become the testa of the mature grain. Thus the harvested grain (Fig 1.1c) contains tissues of different genetic makeup: the embryo contains both maternal and paternal genetic material in equal amounts, the endosperm contains two thirds maternal and one third paternal genetic material, while the seed coat tissues contain only the genetic material of the female parent. These maternally-derived covering structures mean that the harvested product is botanically a fruit or caryopsis rather than a seed. The word grain is commonly used to describe the harvested product, be it caryopsis (wheat) or caryopsis plus husk (barley).

1.4.2 Development of starchy endosperm and aleurone layer.

Wheat, in common with other cereals, is endospermic; that is the endosperm is a persistent storage tissue. (In other plant families, such as the legumes, the endosperm serves as a pool of nutrients for the developing embryo,

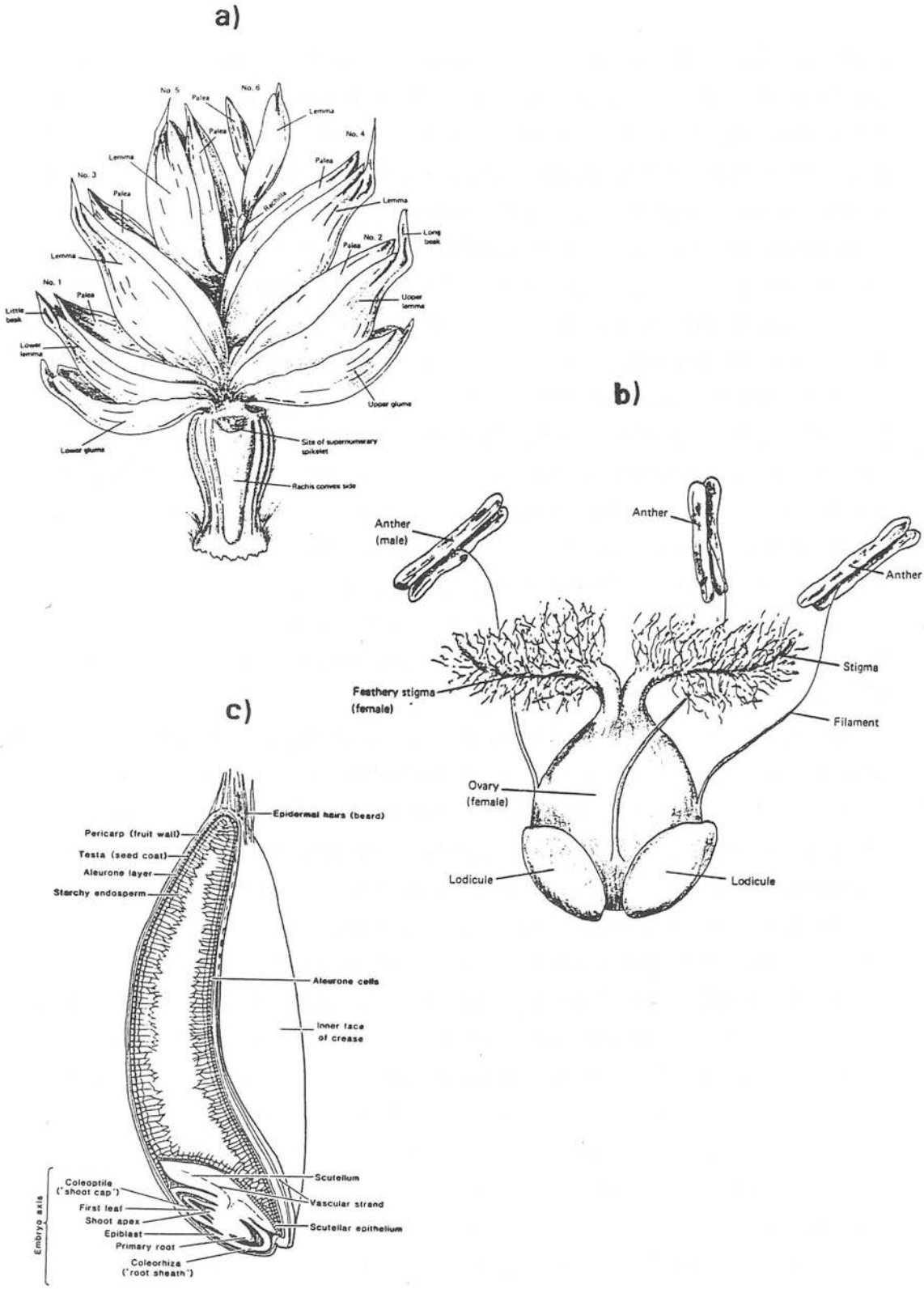


Fig. 1.1 Structure of spikelet (a), flower (b) and mature grain (c) of wheat. (a and b from Hervey-Murray, (1980), c from Barnes, (1989))

becoming depleted and finally resorbed.) The development of the wheat endosperm has been reviewed by Simmonds and O'Brien (1981). Five phases can be identified: 1) fertilization, 2) free nuclear division, 3) cellularization, cell division and some cell enlargment, 4) differentiation of the aleurone layer and accumulation of starch, 5) maturation. The phase of free nuclear division continues for about 3 days after anthesis, and is followed by 24-48 h of cellularization. Cell walls are initially formed by the infurrowing of the embryo-sac wall (Morrison and O'Brien, 1976), and the entire embryo-sac is then progressively cellularized. After cellularization, increase in cell numbers occurs by cell division in the peripheral cells. These meristematic cells divide radially and tangentially. The region of ventral peripheral cells does not divide, but rapidly differentiates into thick-walled cells of granular appearance. Due to the division of the rest of the peripheral layer, this region becomes infurrowed and thus forms the crease of the developing grain. Greater cell expansion of the older cells relative to the peripheral cells also contributes to the formation of the crease. During phase four, from about 14-20 DAA depending on environment, cell division ceases, and increase in grain size is due to cell expansion. Cell division in the peripheral meristematic layer ceases about 14-20 DAA, but the first signs of its differentiation from starchy endosperm to aleurone are apparent from about 10 DAA. Starch granules are present at an early stage, but are lost before maturity. Aleurone cells are fully developed by about 35 DAA and are characterised by thickened cell walls, consisting of an inner, relatively thin layer, and an outer thicker layer. The inner wall appears to be enriched in protein and β–glucan while arabinoxylan and ferulic acid are the major components of the outer wall layer (Fincher, 1989). The cells are tightly packed with aleurone granules surrounded by lipid droplets. During the period of aleurone differentiation the rest of the endosperm cells accumulate starch. Sucrose is thought to be the primary carbon source for starch synthesis and is derived from photosynthesis in the flag leaf and ear. Vascular tissue between the mother plant and the grain is discontinuous, and transfer cells between the rachilla and the crease phloem tissue transport the assimilates. From the phloem in the crease, assimilates pass through the chalazal region, the nucellar projection and into the endosperm cavity and thence to endosperm cells where starch synthesis takes place. Starch deposition begins nearest the source of sucrose, in the crease region. Amyloplasts are present from about 2-3 DAA. Starch is deposited as granules within the amyloplasts. Two types of starch granule have been identified; first-formed are the lenticellular A-type which

grow to 40μm, while the spherical B-type appear later (about 14 DAA) and are less than 10μm in size. Type B granules are more numerous, accounting for 97% of the total granule population, though only about one third of the final starch content. Starch continues to accumulate until maximum dry weight is reached. Protein is also stored, though to a much lesser extent than starch (12-17% of dry weight). Protein bodies are found in the endosperm from about the same time as starch granules are first observed. The maturation phase is associated with loss of water (see below).

1.4.3 Embryo development.

The embryo develops within the embryo sac. During grain development, the embryo is a powerful sink for nutrients, which are initially supplied by hydrolysis of the nucellar parenchyma and later by the hydrolysis of neighbouring endosperm cells (Smart and O'Brien, 1983). The embryo develops rapidly and differentiates into the coleoptile, coleorhiza and scutellum. The scutellar epithelium cells, adjacent to the starchy endosperm, resemble the aleurone cells in containing protein bodies and lipid droplets (Simmonds and O'Brien, 1981).

1.4.4 Control of grain water content.

The relative water content of the grain decreases as the grain develops and gains dry matter, but the absolute water content remains fairly constant from about the stage when starch accumulation begins, until just prior to maximum dry weight. The water potential of the grain is generally lower than the rest of the plant, thereby favouring the influx to the grain of assimilates in solution (Lee and Atkey, 1984). As the absolute water content remains constant there must be a mechanism controlling water uptake and/or water loss from the developing grain. Jenner (1982) suggested that recirculation of water within the caryopsis would allow assimilate uptake in solution and maintain constant water content. However, Lee and Atkey (1984) calculated that evapotranspiration at the surface of the pericarp would enable continuous assimilate uptake, and that a difference of 0.1% in RH between the grain and the surrounding air within the lemma and palea would allow a sufficient rate of evapotranspiration.

1.4.5 Maturation and grain drying.

At, or just before, maximum grain dry weight, the absolute water content of the grain starts to fall rapidly. Meredith and Jenkins (1975) believe this

period of rapid water loss to be independent of weather conditions. They showed that although rain may temporarily increase water content, the underlying drying rate is similar for all cultivars and seasons, and suggest an active process rather than passive desiccation. However, data collected by Dodds and Pelton (1967) over twelve years show that quite different moisture curves were obtained in different seasons. Meredith and Jenkins' data indicate an abrupt cessation of drying at just below 20% moisture (dry basis) and they suggest that this is the point at which harvesting should take place. Dodds and Pelton suggest a slightly lower figure of 17% (dry basis) as the safe stage for combining but their data does not always show a plateau at this level, and in some seasons drying continued to less than 10 % moisture.

1.5 The Control of Germination in Developing and Ripening Grains.

At some stage during grain development the ability to germinate is acquired. However, a period of dormancy usually begins at about the same time, and continues until after harvest, thus ensuring that grains do not normally germinate while still on the ear. The timing and control of these events is of key importance in PHS and will be considered in detail.

1.5.1 Onset of germinability.

Isolated embryos have been shown to germinate from 12 DAA, and yet whole grain germinability is not attained until 26 DAA (Mitchell *et al.*, 1980). This suggests that some factor of the environment in which the embryo is developing prevents precocious germination. The possible role of the plant growth regulator, abscisic acid (ABA) has been much studied. For example, isolated wheat embryos will not germinate if ABA is added to the culture medium (Morris and Bowles, 1987). Viviparous mutants of maize have lower ABA content, or are less sensitive to ABA than normal lines (Kermode, 1990). However, King (1976) has shown that wheat grain ABA levels are quite low at this stage, and do not start to rise until around 25 DAA. It has also been suggested that the osmotic environment surrounding the embryo may prevent its precocious germination (Kermode, 1990). The liquid endosperm generally has low osmotic and water potential (Raghavan, 1986) and it has been shown that culture media of low osmotic potential prevent embryo germination and maintain developmental processes in wheat and barley (Morris and Bowles, 1987).

Gordon (1980), compared a number of "maturation characteristics" in

red and white grained wheat cultivars. His use of the term "embryo maturity" is ambiguous but appears to refer to whole grain germinability rather than isolated embryos. In both red and white cultivars the onset of germinability began at about 30 DAA. There may be difficulties in determining accurately the timing of the acquisition of germinability. Thus, Gordon *et al.* (1979) questioned the assumption that harvest-ripe wheat is germinatively mature and were concerned that dormancy and embryo immaturity were being confused. They carried out "special" germination tests that were designed to overcome dormancy and would therefore give a true indication of whole grain germinability. The "special" tests involved KNO_3, and alternating temperatures (20°C;15°C). Using this measure, they found that acquisition of germination potential began between 10-20 DAA in all cultivars tested, and that the time to reach median germination potential was not significantly different between cultivars. With regard to the possibility of embryo immaturity being present at later stages of grain development, they found that germination potential at harvest-ripeness varied from 50% to 86%. Grain tested without the dormancy-breaking treatments had lower levels of germination, particularly at the earlier sample dates. This could be interpreted to indicate that acquisition of germinability varies within a population, and that published data on dormancy may mask a certain amount of embryo immaturity. However, as the authors concede, not all dormancy breaking treatments were tried. In particular, the fact that the effect of GA_3 was not tested seems a serious omission. Radley (1979) found that application of GA_3 increased the level of germinability of intact grains from 5-6 weeks after anthesis. Gosling *et al.* (1981) noted a similar positive effect of GA_3, and also that stratification could increase germination of immature grains.

The association of the onset of germinability with the beginning of the fall in water content has been well documented and was reviewed by Mitchell *et al.* (1980). In subsequent studies they found that it was actually the drying of the pericarp rather than the whole grain that was significant. In the study by Gordon *et al.* (1979) discussed above it was found that "lifting" of the pericarp enabled germination to occur a day or two earlier. A number of other studies (cited in Mitchell *et al.*, 1980) have shown that the removal of all or part of the pericarp would stimulate germination of immature grains. Wellington (1956a) studied germination behavior during wheat grain development and found that no germination occurred until the green layer "disintegrated". After that, his white grained cultivars could germinate to a high level while the red grained

varieties were still unable to do so (presumably because of the onset of dormancy coinciding with the acquisition of germinability). Following another experiment with the same cultivars, it was suggested that at the early stage the covering layers prevented embryo expansion. Oxygen permeability did not appear to be a major factor (Wellington, 1956b). In seeking an explanation for the "pericarp effect" it is important not to confuse effects on immature grains with those on dormant grains. For example Radley (1980) found that intact grains of the wheat cultivar Capelle/acquired the ability to germinate between 5 and 6 weeks after anthesis, but would germinate (although not to 100%) at 3 weeks if the pericarp was removed. Maris Huntsman germinated at 8 weeks after anthesis if intact and at 4 weeks if the pericarp was removed. The onset of whole grain germinability in this study does appear to be rather later than other data suggest, even taking environmental effects into consideration, and it may be that pericarp effects on suppressing germination are confounded with a seed coat-imposed dormancy. If such a dormancy mechanism began at an early stage, coinciding with the acquistion of whole grain germinability, it would be difficult to separate the effects, except by dormancy-breaking treatments as in Gordon *et al.* (1979) discussed above. The fact that removing the pericarp did not induce 100% germination suggests either that some other dormancy mechanism is operating, or as Gordon *et al.* (1979) propose, there is a degree of embryo immaturity. Rasyad *et al.* (1990) found that onset of germinability began at about 15 DAA in four cultivars; again, later differences in germinability probably reflect differences in dormancy.

Desprez

In concluding this section, it is clear that whole grain germinability is usually acquired at a relatively early stage of grain development, at about the same time as the pericarp loses its green colour. The precise role of the pericarp in preventing germination prior to this is still unknown but may be related to mechanical factors.

1.6 Dormancy.

The evolutionary significance of dormancy is simple to appreciate. Most plant species have evolved dormancy mechanisms to prevent germination until the seed or fruit has been dispersed in time and/or space relative to the mother plant. However, the fact that dormancy is so widespread, that the mechanism(s) has yet to be fully understood, and that genotype and environment can both have considerable effects on its expression all contribute to the difficulty of summarising the literature. There are many, often confusing, definitions of

dormancy. In its broadest sense, dormancy is sometimes used to describe any state of suspended activity, even that of dry seeds lacking only water to initiate germination. Such seeds should not be described as dormant but as quiescent. Villiers (1975) defined dormancy as "a state in which, even though normally favourable conditions of warm temperatures, adequate water and aeration are supplied, growth and development do not take place until a special set of conditions has been experienced". Adding to the confusion is the division into different types of dormancy. Thus, Belderok (1961) referred to "absolute dormancy", which was "impossible to break", and "relative dormancy" or "delayed germination" which could be overcome by various dormancy-breaking treatments, but then included both types in his collective definition of dormancy. Bewley and Black (1982) attempted to clarify the situation and described three types of dormancy, outlined below:

1) Dormancy or primary dormancy.
A seed which is dormant over a range of normal temperatures, but germinates after several weeks prechilling. Sometimes referred to as innate dormancy (Roberts, 1972) due to its development whilst on the mother plant.
2) Dormancy or relative dormancy.
A seed which is dormant at temperatures above a certain value. Sometimes included in primary dormancy (eg. Koller *et al.*, 1962), or called enforced dormancy (Roberts, 1972) due to the fact that it is imposed by an environmental limitation.
3) Secondary dormancy.
A seed which, after exposure to certain environmental conditions eg light, high/low temperature, excessive moisture, fails to germinate in favourable conditions. Referred to as induced dormancy by Roberts (1972).

Dormancy may also be categorised according to the site of the dormancy mechanism. Thus embryo dormancy refers to some factor in the embryo itself preventing germination, while coat-imposed dormancy refers to the type of dormancy that can be overcome by the removal of the seed coat. Sometimes, both types of dormancy may be present in the same seed. Dormancy may be quantified in different ways. Thus, the proportion of dormant grains at a particular point in time may be recorded, and/or the duration of dormancy.

Most cereal species are dormant to some degree. Common in cultivated varieties is a temperature-dependent relative dormancy in which dormancy is

only apparent at temperatures above about 18°C. Relative dormancy may completely prevent germination at some temperatures, while only slowing down the rate of germination at others. Secondary dormancy, induced by low temperature and high relative humidity, has been demonstrated in wheat crops left in the field (Belderok and Habekotté, 1980). Black *et al.* (1987) showed that removal of seed coats or other surgical treatments allowed germination of embryos from dormant grain. However, the embryos germinated at a slower rate than those from non-dormant grains, and still showed temperature dependence, with a reduced rate of germination at warmer temperatures, and it was thus suggested that both embryo and seed-coat dormancy were present.

1.6.1 Embryo dormancy.

Embryo dormancy refers to the state whereby fully developed embryos will not germinate on isolation. In reviewing cereal dormancy Belderok (1961) found few instances of embryo dormancy but more recent studies have indicated wider occurrence. Radley (1980) showed that isolated embryos of Maris Huntsman wheat had lost their earlier ability to germinate by about 6-8 weeks after anthesis, and King (1982) presents similar findings for various wheat cultivars. Some sort of embryo dormancy is probably involved in the few cases where white grained cultivars have exhibited dormancy (see discussion of genotypic effects below). The mechanism of embryo dormancy is not yet understood. Belderok (1961) discounted the theory that embryo dormancy was due to insufficient nutrients to support germination. In the same review he wrote that "growth-promoting and growth-inhibiting substances (have) hardly ever been investigated (in relation to) the phenomenon of dormancy". In the years since that review was written a considerable amount of research effort has been devoted to the possible role of ABA in embryo dormancy. Exogenous ABA inhibits germination of isolated embryos (Stoy and Sundin, 1976). King (1976) reported that ABA begins accumulating in developing grains during the latter part of the linear growth period until maximum dry weight is reached; a rapid decline is then observed at the time of grain water loss to reach a low level at harvest. Embryo and grain germinability decreased as ABA increased. However, these are correlations and are no indication that ABA is in fact responsible for embryo dormancy. In fact there are no consistent correlations between level of dormancy and grain or embryo ABA content in dormant and non-dormant varieties of wheat (Walker-Simmons *et al.*, 1990). However, a role for ABA cannot be ruled out. Dormant wheat embryos are apparently more

sensitive to ABA than non-dormant ones (Walker-Simmons, 1987), and environmental effects on dormancy may be related to environmentally-mediated effects on ABA sensitivity.

GA_3 is a commonly used dormancy-breaking treatment (International Seed Testing Association, 1976) but there is no evidence that dormant embryos contain less GA than non-dormant ones, or differ in sensitivity to GA.

The possible role of respiratory metabolism in embryo dormancy has been considered by Black *et al.*(1987) and also by King (1989). Various respiratory inhibitors, high oxygen concentrations, nitrate and nitrite can break dormancy (Roberts and Smith, 1977). However, earlier theories (Roberts, 1969) that the activity of the pentose phosphate pathway (PPP) is low in dormant seeds, and increases when dormancy is broken have not been proven. Likewise, there is no evidence that the balance between CN-resistant and CN-sensitive respiration is involved in cereal dormancy (Black *et al.*, 1987).

Other theories of embryo dormancy were reviewed by Black *et al.*, (1987). Studies of temperature-dependent relative dormancy have led to proposals that such temperature effects may be related to cell membrane properties. Finally, it may be that dormant embryos are unable to express certain key genes necessary for synthesis of germination enzymes.

1.6.2 Coat-imposed dormancy.

This type of dormancy is exhibited by wheat and other cereal species. The mechanism whereby the seed coat imposes dormancy has not been fully determined. There are at least six possibilities identified by Bewley and Black (1982):

1) Interference with water uptake.
2) Interference with gaseous exchange.
3) Germination inhibitors in the seed coat.
4) Prevention of leakage of inhibitors from the embryo.
5) Modification of light reaching the embryo.
6) Mechanical restraint.

In cereals in general, and wheat in particular, it is likely that 2, 3 and 6 are the most important. Thus, coat-imposed dormancy may be due to competition for oxygen. The hull of barley and the seed coat of wheat contain large amounts of phenolic compounds. Oxidation of these compounds may prevent the necessary oxygen for germination from reaching the embryo (Côme *et al.*, 1988). Support

for this hypothesis is given by the fact that germination is increased by raising the concentration of oxygen. Dormant barley seeds absorbed significantly more oxygen than non-dormant seeds during the first hours of imbibition, mainly due to greater oxygen absorption by the hull (Côme *et al.*, 1988). More oxygen was absorbed at higher temperatures. No qualitative or quantitative changes in phenolic compounds or polyphenol oxidases were observed during dry storage of barley, but the oxidation reaction, whereby the hull uses up the available oxygen, was delayed (Corbineau *et al.*, 1984, cited by Côme *et al.*, 1988). It was suggested by Côme *et al.* (1988) that this would allow sufficient time for germination to begin. They also suggested that the seed coat of wheat may act in a similar way. However, no differences in oxygen consumption during imbibition have been detected in dormant and non-dormant wheat cultivars (Miyamoto *et al.*, 1961). Studies by Durham and Wellington (1961) indicated that isolated wheat embryos will germinate to a high level at 5% oxygen, and that some will germinate at 0.5% or less. Bewley and Black (1982), reviewing seed coat effects on oxygen uptake, suggest that though there may be sufficient oxygen for respiration, it is not enough to repress the production of germination inhibitors, or oxidise germination inhibitors in *Sinapis arvensis*, and *Xanthium pennsylvanicum* respectively. Similar studies on wheat would be useful.

Seed coats of many species contain germination inhibitors. In wheat, the major inhibitory chemicals are catechins and tannin-like compounds, and, to a lesser extent, alkaloids and others unidentified (Miyamoto *et al.*, 1961). Isolated embryos from dormant wheat grains were found to be more sensitive to catechin-tannin extracts than were embryos from non-dormant grains (Stoy and Sundin, 1976). Gordon (1979) questions whether catechin-tannins act as germination-inhibitors *in vivo* and suggested that their effect may be related to interference with oxygen supply as discussed above.

It has been suggested that differences in dormancy in white and red grained wheats are related to differences in mechanical resistance of the seed coat (Wellington, 1956b). A later study suggested that the more wrinkled epidermis of the white grains imposed less resistance to water uptake and embryo expansion. (Wellington and Durham, 1961). However, their results may reflect differences in embryo growth potential rather than differences in mechanical strength of the seed coat. In addition, it is possible that the results were confounded by differences in catechin-tannins between the red and white grains. Our understanding of cereal dormancy is further complicated by both genotypic and environmental effects on the onset, intensity and duration of

dormancy.

1.6.3 Genotypic effects.

In wheat, genotypic effects are most apparent between red- and white-grained cultivars. Red-grained varieties have long been known to possess greater dormancy than white-grained ones (Nilsson-Ehle, 1914). It is now thought that this association is pleiotropic, i.e. that the red phlobaphene pigment or its precursors, catechin and catechin-tannins, are responsible for the observed dormancy (Gale and Flintham, 1988). Red-grained cultivars were found to be more dormant, and contained almost twice as much catechin-tannin-like compounds as white cultivars (Miyamoto *et al.*, 1961). Alleles at three loci control grain colour (Metzger and Silbaugh, 1970) and the effects are probably additive, explaining some of the variation within red-grained wheats with respect to dormancy. However, even some three gene red wheats are less dormant than others (Noll *et al.*, 1982; Czarnecki, 1987) and it has been suggested that there may be genetic differences in sensitivity to the inhibitory effect (Gale and Flintham, 1988). Alternatively, of course, there may be other dormancy mechanisms in operation in addition to that associated with coat colour. Other genes that affect the duration of dormancy, even within white wheats, have been identified. A white-grained line, Kenya 321, was found to have a recessive gene for dormancy, thereby limiting its potential use in breeding programmes (Bhatt *et al.*, 1983). Screening of over 3000 white grained accessions in Australia identified 20 lines with a good degree of resistance. The genetic basis is, as yet, unknown (Mares, 1987a). Genotypic variation for dormancy is the major source of resistance to PHS, but is not thought to be involved with resistance to PMAA.

1.6.4 Environmental effects.

The many factors encompassed in the word "environment" - temperature, rainfall, humidity, daylength, radiation, windspeed - indicate how difficult it is to correlate a particular environmental variable with its precise effect on dormancy. Environment x genotype interactions further complicate any investigation. The general pattern appears to be that warmer temperatures during grain development reduce the intensity and duration of dormancy. Strand (1989) reported on the results of a twenty year research programme in which temperature, global radiation, rainfall, RH and rainfall/temperature ratio were correlated with measured dormancy in spring wheat. His results show that

higher temperature and more intense global radiation in the pre-harvest period did reduce dormancy. Also, higher rainfall, RH and rainfall/temperature were associated with increased dormancy. However the environment x genotype interactions showed that temperature had no effect on the dormancy of one cultivar, while moisture-related variables did, but only when dormancy was measured at 10°C. This perhaps suggests that there might be two dormancy mechanisms operating, and that the relative dormancy more apparent at 20°C was not influenced by moisture-related factors but that an additional dormancy, still present even at the lower germination temperature, was. Little work has been done to reconcile these environmental effects with the dormancy mechanisms discussed above. Ideally, individual environmental factors should be studied in controlled environment conditions for their effects on, say, content of inhibitory compounds in the seed coat. Thus, if high temperatures decrease dormancy, we might expect lower levels of inhibitors.

After-ripening may be defined as the period during which dormancy is lost. In cereals, the duration of after-ripening may vary from a few days to several months. Environmental effects on after-ripening have been extensively studied. Gale *et al.* (1983) showed that rapid drying reduced the duration of dormancy. Separate effects of temperature and moisture-related factors, both of which affect drying rate, have also been investigated. Hagemann and Ciha (1987) demonstrated that storage at higher temperature resulted in an accelerated loss of dormancy. High seed moisture during the after-ripening period increased the level of dormancy in a red-grained cultivar, but reduced dormancy in a white-grained one (Skinnes and Sorrels, 1990). The physiological effect of the environment during after ripening has been postulated as a reduction in ABA, but there is no evidence to support this (King, 1989).

1.7 Alpha-amylase in Grain Development and Germination.

That alpha-amylase is the major cause of sprouting damage has been previously discussed. Two possible sources - PHS and PMAA - of the enzyme in harvest-ripe grains have been identified, and will be further discussed in the following sections.

1.7.1 Alpha-amylase in developing grains.

Alpha-amylase is normally present during the early stages of wheat grain development. Olered (1963) found high levels of alpha-amylase in immature wheat grains, and showed that the levels fell rapidly as the grain matured. This

early enzyme production has become known as "green" amylase due to the fact that it is mainly located in the pericarp, and disappears as the green colour is lost. The gene locus *α-Amy 2*, located on the long arms of chromosomes 7A, 7B and 7D (Lazarus *et al.*, 1985), controls production of a group of "green" enzymes found mainly in the pericarp and, to a lesser extent, in other tissues. The group is variously referred to as GI (Marchylo *et al.*, 1980), Group 2 (Sargeant, 1980) and I (Daussant *et al.*, 1980) and has a pI of between 4.5 and 4.8. Marchylo *et al.* (1980) identified another group of isoenzymes in immature wheat grains, with an intermediate pI and which they called GII. It appears to be of minor importance, is found in very small amounts, mainly in the endosperm, and contributes little to the total alpha-amylase activity. Gale (1983) suggested that this group was almost certainly also controlled by *α-Amy 2*. *α-Amy 3* controls expression of the more-recently described third group of "green" isozymes, which have a much higher pI of about 10 (Daussant and Renard, 1987; Baulcombe *et al.*, 1987). They are mainly located in the pericarp, first appear about 11 DAA and then decline rapidly. The role of the various "green" amylases is thought to be in the degradation of starch granules present in the immature pericarp (Hill and MacGregor, 1988). The enzyme is then thought to be destroyed, and very little, if any, is present in the mature grain. However, it is possible that drought, or other causes of premature shrivelling, might result in dried, immature grains and measurable levels of alpha-amylase in harvested samples. This is possibly the reason for the lower HFN noted in samples from take-all infected plots (Bateman *et al.*, 1990). Thus, harvest-ripe grains are normally characterized by low levels of alpha-amylase, dependent on cultivar (Kruger,1989), and activity will only increase due to PHS and/or PMAA.

1.7.2 Alpha-amylase in germinating grains.

Alpha-amylase synthesis in germinating cereal grains has been one of the most intensely studied areas of plant physiology/biochemistry, particularly with respect to the control of alpha-amylase production. Due to the obvious difficulties involved in studying a largely unpredictable phenomenon that, by definition, occurs only in unharvested grains, much of our knowledge on alpha-amylase in germinating grains is based on laboratory-germinated rather than sprouted samples. This has disadvantages, as Meredith and Pomeranz (1985) noted: "the physical conditions and the resulting physiologic changes differ.....it is dangerous to transfer conclusions about one kind of germination....to another". There are many obvious differences between PHS and laboratory

germination, including age of grains, attachment to the parent plant, and uncontrolled environmental conditions. In addition, few laboratory studies have used varieties susceptible to PHS. In fact, much of the literature on GA control of alpha-amylase synthesis is based on studies of the rare naked barley cvHimalaya, and on isolated aleurone layers rather than intact grains. Nevertheless, lab studies are justified "in the hope that by understanding (lab germination), which is much more convenient experimentally, key features can be identified which can then be investigated in grain which is prematurely germinating" (Chandler *et al.*, 1987).

The initial sources of energy in germinating grains are the sugar reserves in the embryo; these are depleted over the first 24 hours of imbibition (Bewley and Black,1983). After that, energy for embryo growth and development is provided by the mobilization of the starch reserves in the endosperm. The procedure may be summarised as follows: GA diffuses from the embryo to the aleurone layer where it initiates the synthesis of alpha-amylase, which in turn is secreted into the starchy endosperm to begin starch granule hydrolysis. Undoubtedly, though, this is an oversimplification of a complex procedure that is not yet fully understood. It has long been recognized that the presence of the embryo is essential for reserve mobilization (Brown and Morris, 1890), and the findings that a) exogenous GA can substitute for this requirement (Paleg, 1960) and b) isolated embryos release GAs (Radley, 1967) have led to conclusions that embryo-derived GA triggers reserve mobilization *in vivo* (eg. see Bewley and Black, 1983; King, 1989,). However, there are contradictory findings discussed in reviews by Halmer (1985), Brearley *et al.* (1987), Hill and MacGregor (1988), and Fincher (1989). The exact site of GA synthesis is unknown and there is still no direct evidence for the diffusion of GA from the embryo to the aleurone. There is also evidence suggesting that the embryo supplies another factor required for alpha-amylase synthesis. Thus, de-embryonated barley synthesised less alpha-amylase than whole grains, but application of GA_3 only raised activity to 40% of that in whole germinating grains (Marchylo *et al.*, 1987).

Chandler and Jacobsen, (1991) demonstrated an increase in mRNAs coding for alpha-amylase in GA_3-treated isolated barley aleurone layers indicating that increases in alpha-amylase are due to *de novo* synthesis rather than release of existing forms. (The caution against assuming similarities between different systems is well illustrated by this study. Thus, in what is thought to be the first comparison of alpha-amylase mRNAs in isolated

aleurone layers and germinating grains, it was found that although aleurone from germinating grains expressed the same groups of mRNAs as GA_3-treated aleurone layers, the timing of expression of the different groups varied.) Exactly how GA might activate the gene is unknown and receptor sites have not been identified (Fincher, 1989). Alpha-amylase synthesis is thought to occur in the rough endoplasmic reticulum of the aleurone cells and is transported via transport vesicles to the plasmamembrane where it is secreted into the starchy endosperm (Brearley *et al.*, 1987) via channels in the outer cell walls of the aleurone layer (Fincher, 1989). Alpha-amylase secretion appears to be under GA control, although independent of synthesis. GA-control of secretion is possibly via induction of functional proteins such as the enzyme β-1,3-glucanase (Brearley *et al.*, 1987) required to modify the inner cell wall of the aleurone prior to alpha-amylase release. Ca^{2+} appears to be a requirement for secretion of high pI forms of alpha-amylase (Hill and MacGregor, 1988) and possibly plays a role in fusion of secretory vesicles with the plasma membrane (Fincher, 1989).

Alpha-amylase produced during germination consists of two groups of isozymes. The main group, collectively known as "malt" enzymes (or Group 1 (Sargeant, 1980), GIII (Marchylo *et al.*, 1980), or II (Daussant *et al.*, (1980)) have pIs of about 6.0-6.5 and are controlled by the α-*Amy 1* gene locus, probably located on the long arm of chromosomes 6A, 6B and 6D (Gale & Flintham, 1988). A set of α-*Amy 2*-controlled low pI enzymes, similar to the "green" isozymes are also expressed, but to a lesser extent. Marchylo *et al.* (1987) showed that more than 84% of the alpha-amylase activity in germinating wheat was due to "malt" isozymes. The proportion decreased with time, accompanied by an increase in low pI forms. The two groups of isozymes appear to be differentially regulated. Thus, high pI isozymes increased 20 fold in GA_3-treated half grains of wheat, while low pI forms only increased 4 fold. In germinating barley, embryo removal increased the relative proportion of low pI forms (Marchylo *et al.*, 1987), indicating less dependence on GA of these isozymes compared to the high pI ones.

Previously, it was believed that aleurone cells were the only site of alpha-amylase production, but in recent years there has been considerable debate as to the role of the scutellum in synthesis and secretion of alpha-amylase (see Palmer, 1989; Briggs, 1987). While the aleurone as a source of alpha-amylase is not in doubt, what is questioned is the contribution, if any, of the scutellum. Again, much of the relevant work has been done with barley rather than wheat.

In germinating barley, alpha-amylase was initially detected adjacent to the scutellum, and moved with time in a front parallel to the scutellum. This was interpreted as showing that the scutellum was the initial, quantitatively important, source of alpha-amylase (Gibbons, 1981). However, Palmer (1989) believes that this pattern of endosperm breakdown can be explained by assymetric transport of GA from the embryo to the aleurone, with cells closest to the embryo responding first and the alpha-amylase they produce then spreading along the junction of the embryo and endosperm. Further, he argues that contamination by aleurone tissue could account for apparent scutellar alpha-amylase production. Incubated isolated barley embryos synthesised low pI amylase, and did not respond to GA_3 (MacGregor and Marchylo, 1986) which is contrary to what would be expected if contaminating aleurone tissue was present. However, Palmer (1989) points out that aleurone tissue does produce a small quantity of low pI amylase. Mares, (1987b) compared alpha-amylase production in incubated wheat embryos with that of intact grains incubated under identical conditions. The initial rate of increase of activity in embryos was equal to or greater than that of intact grains. Although there was contamination by aleurone cells, the observation that isolated embryos did not respond to GA_3, and that GA-insensitive lines had the same initial pattern as GA-sensitive ones, led Mares to conclude that the embryo-scutellum was the initial source of alpha-amylase. Further studies, in which embryos were removed from incubated grains at progressively later stages, indicated that embryo-derived alpha-amylase was synthesised from about 12h while the aleurone began synthesising alpha-amylase at about 36-40h (Mares, 1987b). The studies discussed above indicate that barley and wheat embryos do not respond to exogenous GA_3. However, Chandler and Mosleth (1990) noted that the scutellum is capable of synthesising GA (Radley, 1967) and questioned whether the apparent lack of response might be due to already-saturating endogenous levels of GA. They carried out primer extension studies on mRNA extracted from scutellar tissue of barley previously imbibed in different media ($\pm$GA, $\pm$ Paclobutrazol, an inhibitor of GA synthesis). Their results indicate that the scutellum is able to express high pI alpha-amylase transcripts and that this expression is regulated by GA. However, the study did not investigate the expression of low pI alpha-amylase, which is thought to be the most important component of embryo alpha-amylase during germination. Garçia-Maya *et al.* (1990) found only low pI alpha-amylase in older (>40 DAA) isolated wheat embryos, and that expression of low pI alpha-amylase (in 30 DAA embryos)

appeared to be independent of endogenous GA_3. Furthermore, both ABA and osmotically active media had little effect on expression of low pI alpha-amylase.

Whatever the precise role of the embryo-scutellum in alpha-amylase synthesis, the size difference between the tissues of the embryo-scutellum and the aleurone mean that the aleurone is quantitatively the most important source of alpha-amylase during germination and, by implication, in malting. However, the debate is very relevant to PHS. Gale and Marshall (1975) observe that "sprouting does not often proceed beyond 1 or 2 days", and Mares (1987b) suggests that scutellar enzyme production during the early stages of germination would be sufficient to downgrade wheat.

1.7.3 Alpha-amylase in sprouted grains.

The assumption is made that similar processes to those described above take place when PHS occurs in the field. Certainly, the amylases found in sprouted grain appear to be the same as those found in normally germinated grain (Sargeant, 1980). Furthermore, assumptions that PHS involves a similar GA-mediated aleurone response to that occurring during normal germination are supported by findings on so-called aleurone dormancy. The aleurone of developing grains is normally insensitive to GA, i.e. de-embryonated immature grains do not produce alpha-amylase in response to exogenous GA (Black *et al.*, 1983), but Gale *et al.* (1983), recorded germinability and GA-sensitivity during grain development and found that a short period of whole grain germinability (when, presumably, PHS would have been possible) coincided with a period of aleurone sensitivity.

1.7.4 Prematurity alpha-amylase (PMAA) in unsprouted grains.

If little is known about the control of alpha-amylase in sprouted grains, even less is known about PMAA. Marchylo *et al.*, (1980) were surprised to find small amounts of "malt" high pI alpha-amylases in developing wheat grains. They were present in all cultivars tested, predominantly in the endosperm, and generally increased in activity during grain maturation. Sargeant (1980) found that high pI isozymes appeared by 35 DAA in unsprouted grains of Champlein wheat and increased in intensity until maturity. The few studies on the location of PMAA report different results. In Maris Huntsman wheat, activity was first seen, and accumulated more rapidly at the embryo end of the grain (Gale *et al.*, 1987). In contrast, Cornford *et al.* (1987) found that wheat cvFenman produced alpha-amylase in close association with aleurone tissue in the ventral portion of

the grain. Fenman and Maris Huntsman are related, but an unrelated Australian cultivar, Spica, was shown to produce alpha-amylase more uniformly throughout the endosperm (Mares and Gale, 1990). In all of these studies the alpha-amylase detected was the high pI, germinative or "malt" form of the enzyme.

MacGregor and Dushnicky (1989) have shown that the crushed cell layers adjacent to the scutellum of developing barley kernels contains low levels of alpha-amylases similar to those characteristic of germination. Degradation of starch granules was observed in cells adjacent to the crushed cell layer from 14 DAA, suggesting that the role of alpha-amylase was to initiate starch breakdown, to supply products for embryo growth. It was suggested that whatever triggers this alpha-amylase synthesis in barley, may also be responsible for PMAA observed in wheat (MacGregor and Dushnicky, 1989). Marchylo and Kruger (1987) studied starch degradation in maturing grains of four wheat cultivars. None of the cultivars showed any indication of sprouting but starch granule degradation adjacent to the crushed cell layer was evident from about 38 DAA in one variety and from about 44 and 49 DAA in two other varieties. The early part of grain development was not studied, neither was the crushed cell layer itself specifically examined, so the results are not strictly comparable with the barley study discussed above (MacGregor and Dushnicky, 1989). However, at the earliest sampling (30DAA) there was no indication of starch degradation, indicating that PMAA is probably unrelated to any earlier alpha-amylase associated with the crushed cell layer.

In attempting to determine the mechanism involved in PMAA we are faced with similar problems as when laboratory germination is compared with field sprouting, and are forced into using a well-researched system to infer similar mechanisms in a system of which we know very little. Thus, while Hill & MacGregor (1988) write that "the aleurone layer of some cereal grains appears to become activated for a period of time during kernel development and produce germination-type alpha-amylases" there is only limited evidence to support this. Gale *et al.* (1983) reported that a "window" of aleurone-sensitivity occurred at about the time when PMAA was observed. However, Marchylo and Kruger (1987) having identified starch degradation adjacent to the crushed cell layer of mature unsprouted wheat, examined the embryo as the possible source of alpha-amylase. They found that more than 50% of alpha-amylase activity in germinating embryos was due to low pI forms, and that about 40% of the (very low) level of activity in mature unsprouted grains was also low pI. This is in

contrast to the analyses of PMAA reported above, in which high pI isozymes predominated. It should be noted that attempts to relate the location of PMAA to its source of synthesis are open to the same arguments discussed above in relation to the initial source of alpha-amylase in germinating grains.

In an early study to investigate the inheritance of susceptibility to PMAA Bingham and Whitmore (1966) suggested that one, or at most, two, duplicate recessive genes were responsible. In the case of Spica, Mares and Gale (1990) believe that a single recessive gene, possibly on chromosome 6B, is responsible. It is clear however, that both genotype and environment are important in the expression of PMAA, with adverse climatic conditions amplifying the genotypic expression.

There is evidence that grain drying rate influences PMAA. In a growth chamber experiment, in which "fast" and "slow" drying rates were produced by relative humidities of 56% and 92% respectively, alpha amylase activity increased to a significantly higher level in the slow drying treatment. It was suggested that the effect was due to either an extension of the risk period or to a direct effect of drying rate. Such an effect, it was suggested might be mediated via the effect of drying rate on aleurone responsiveness to GA_3 (Gale *et al.*, 1983). This was based on a study showing that slow drying of detached grains increased the potential for alpha-amylase production (indicated by a shorter incubation period required for response to occur) and rapid drying reduced it (King and Gale, 1980). However, genotypic variation in response was noted and Maris Huntsman was found to produce excessive alpha-amylase whether dried rapidly or slowly. In contrast, in intact plants, faster drying did reduce PMAA (Gale *et al.*, 1983). This suggests that, if drying rate is important, it is more likely to be an indirect effect via an extension of the risk period. There is circumstantial evidence in support of the hypothesis that slow drying increases PMAA. For example, late fungicide treatment reduces HFN (Stevens *et al.*, 1988). Fungicide treatment is correlated with increased duration of green leaf area, and increased grain moisture. It was suggested that high levels of disease accelerate natural senescence, allowing the plant to ripen and mature in better weather conditions of July or early August. There is also evidence against the "drying rate" theory. Firstly, a study of farmers' crops in 1988 revealed no correlation between rate of moisture loss from 40-20 % moisture, and HFN at harvest (Kettlewell and Astbury, 1990). It may be significant that the year was a "good" one, and that HFN's were generally high, indicating little alpha amylase activity. Alternatively, it may be that the varieties concerned - Mercia and

Avalon - are not susceptible to PMAA. Avalon, though, has been the most important breadmaking variety for several years, and did have reduced HFN in 1985 and 1987, and relatively little visible sprouting. Secondly, several experiments have shown that, while the duration of green leaf area is prolonged by spring fertiliser nitrogen application, HFN is increased (Stevens *et al.*, 1988). It is difficult to reconcile these results with the effects of fungicide reported above, although it may be, that while fungicide application only affects drying rate, nitrogen has other effects. For instance, the duration of grain growth is lengthened, and this may in turn delay or shorten the particular stage of grain ripening at risk from PMAA.

A possible relationship between GA-sensitivity and PMAA is indicated by a study using near-isogenic lines of Maris Huntsman, in which it was found that the presence of the GA-insensitive dwarfing gene (*Rht3*) reduced both the numbers of grains with PMAA, and the relative amounts of activity in these grains, compared to the semi-dwarf (*Rht1*), and the normal, tall, line (*rht*) (Gale *et al.*, 1987).

Finally, the possible areas of confusion between the different sources of alpha-amylase in harvest-ripe grains should be clarified. The phrase "incipient sprouting" is occasionally used in the literature, apparently to describe grains in which germinative alpha-amylase synthesis has begun prior to visible sprouting. Thus, in a study discussed above (MacGregor and Kruger, 1987) it was suggested that "incipient sprouting" had occurred in a variety that showed starch degradation, high levels of germinative alpha-amylase activity, virtually no dormancy, but was not visibly sprouted. "Incipient sprouting" would appear to be distinguishable from PMAA by the fact that PMAA is not necessarily associated with susceptibility to PHS, whereas "incipient sprouting" tends to occur in susceptible varieties possessing low dormancy.

The low HFN's recorded in the UK in 1977 were due to a high level of PHS (Home-Grown Cereals Authority, 1977). In contrast, similarly poor HFN's in 1985 and 1987 were associated with much lower levels of sprouting (Home-Grown Cereals Authority, 1985; 1987). The causes have been attributed to PMAA in 1985, and pre-dormancy PHS in 1987 (Gale and Flintham, 1988). There is little direct evidence for these conclusions, however. Pre-dormancy PHS was observed at approximately five weeks after anthesis in 1987 trials with Bersee and Maris Huntsman (Flintham and Gale, 1990), but there are no reports of this type of PHS on a national scale in that year. In the Home-Grown Cereals Authority surveys sprouting is scored as present or absent (Cranstoun,

pers. comm.) and it is impossible to determine when the observed sprouting took place, or how advanced it was. Given the different spectrum of varieties in 1985 and 1987 compared to 1977, the results could be explained by genotypic variation in either visible sprouting, and/or rate of alpha-amylase production. Barley genotypes differ greatly in alpha-amylase production during germination (Raynes and Briggs, 1985). Thus, one genotype with a particularly high (or low) capacity for alpha-amylase production could be responsible for the lack of correlation between sprouting and HFN recorded in the three years. While it is undoubtedly true that some varieties are susceptible to PMAA its widespread occurrence in 1985 is open to question, and the low HFNs in 1985 may have been due to so-called incipient sprouting as discussed above. That PMAA is distinct from PHS is indicated by studies showing that varieties which are resistant to PHS may nevertheless have high levels of alpha amylase (Mares, 1987a). Rankings for PHS do not correspond with rankings for PMAA, although some varieties do appear to be susceptible to both (Morgan, 1988).

It is clear from the above discussion that our knowledge of the factors affecting PMAA is limited. In particular, the mechanism of the environmental effects involved in PMAA has not been determined. In addition the physiological and biochemical basis of susceptibility and resistance is unknown. This thesis examines some of the environmental and genotypic factors involved in PMAA.

2. Environmental and Genotypic Effects on Alpha-amylase Activity During Wheat Grain Development: field trials, 1989 and 1990.

2.1 Introduction.

There appear to be three possible causes of excessive alpha-amylase activity in harvested wheat grains. In 1977, there was a high level of post-dormancy PHS, and alpha-amylase produced during germination led to low HFN's. The equally low HFN's recorded in 1985 and 1987 have been attributed to PMAA, and pre-dormancy PHS, respectively (Gale and Flintham, 1988). However, evidence for pre-dormancy PHS in 1987 is not particularly strong since it was observed in only a limited number of field trials (Flintham and Gale, 1990) and Gale and Flintham (1988) themselves write that "the phenomenon has rarely been observed". Nevertheless, their results appear to have been interpreted by some (eg. Hough, 1990), as an indication that pre-dormancy PHS was of widespread occurrence. In fact, national data show that the incidence of sprouting was similar in 1985 and 1987 at 0.63 % and 0.74 % respectively (Home-Grown Cereals Authority, 1985, 1987), and the conclusion of Morgan (1988), that PMAA was the major cause of low HFN's in both years, thus seems more appropriate. National data do not distinguish between pre- and post-dormancy sprouting and there is also the possibility of overlap between PMAA and PHS (see section 1.7.4). In any investigation of the control of alpha-amylase production, it is clearly essential that the physiological origin of the enzyme be identified, and that confusion in terminology is avoided as far as possible.

PHS and/or PMAA has been a problem in three of the last fourteen years in the UK. Such an unpredictable pattern makes it difficult to carry out field-based investigations, and laboratory germination and rain simulator studies have provided much of the current knowledge about the physiological background to alpha-amylase production. When field experiments are carried out, the diverse environmental variables mean that any one factor can rarely be correlated with a measured grain response with a high degree of precision. However, until there is a better understanding of the exact environmental conditions leading to PHS and PMAA it is impossible to simulate these conditions in a controlled environment. In order to study this phenomenon adequately it is essential to use large, uniform plant populations. This is because (1) there is much variation, both within and between ears, (2) time course studies of grain development require many plants, (3) it is desirable to

include resistant and susceptible genotypes. Hence the present studies have been carried out mainly under field rather than controlled growth conditions.

One environmental variable implicated in the onset of sprouting damage may be the grain drying rate. For instance, Gale *et al.* (1983) suggested that the length of time taken for grain to dry from 40% to 20% moisture (wet basis) could affect the levels of prematurity alpha-amylase produced. Correlations have been sought between measured drying rate and HFN for commercial field crops in different locations, having a range of drying rates, (Kettlewell and Astbury, 1990), but no field experiments directly comparing drying rates have been reported. The work reported here is an attempt to investigate the possible role of grain drying rate on the production of alpha-amylase during grain maturation. A partly-controlled environment was imposed on field plots of different cultivars in an attempt to induce different drying rates and thereby examine environmental and genotypic effects on sprouting damage, be it a result of PHS or PMAA.

2.2 Materials and Methods.

2.2.1 Plant material (1989)

Four winter wheat cultivars (Apollo, Avalon, Fenman and Mission) were drilled as single plots (22m x 2m) on 11th October 1988 in sandy loam soil at March Park field, part of the Bush Cereal Trials Centre. Previous cropping was winter barley (five years). Seed rate was 200 kg/ha and P_2O_5 and K_2O (80 kg/ha each) were applied on 4/11/88. Top dressings of 50 kg/ha and 110 kg/ha N were applied on 17th March and 24th April respectively. Weed and disease control was as follows:

Herbicides:	Swipe	13/4/89
Fungicides:	Mistral	2/5/89
	Sportak + Patrol	29/5/89
	Tilt Turbo + Bravo + Bavistin	20/6/89
Growth Regulator	5C Cycocel	1/5/89

Treatments

Treatments were applied to one third of each plot from 29 DAA. Plots were been initially checked for uniformity, using plant height as a variable. The treatments were as follows:

Wet: Protected from rain by a polythene "tent" but wetted daily with 2.2 l/m^2 of water applied by knapsack sprayer.

Covered: Protected from rain as above (Plate 2.1).

Control: Untreated

The intention was to continue these treatments until harvest, but strong winds prevented this, and the "tents" were removed from both the wet and the covered areas at 39 DAA. The daily wetting treatment was continued on the previously covered and wetted area, with the aim of slowing the grain drying rate; the previously covered area continued to be sampled. Plots were regularly sampled from 7 DAA until harvest (66 DAA). Measurements were made of fresh and dry weight, germinability and alpha-amylase activity.

Plate 2.1 Polythene tents used in the covered treatment.

2.2.2 Fresh and dry weight measurements (1989).

Ten ears per treatment were sampled twice weekly. They were placed in polythene bags inside an insulated cool box. Within approximately 1h of sampling, three grains from the outer florets of the central spikelets were removed, and the ears were then stored at -18°C for later determination of alpha-amylase activity. The grains removed were bulked and 10 grains placed into each of three pre-weighed and pre-dried glass vials. Fresh weight was recorded, and the samples dried to constant weight at 80°C. Dry weight was recorded and water content and percentage moisture (wet basis) calculated. At each sample date the physiological age ("DAA") was determined using the developmental scale described by Riffkin (1987) using wheat (*Triticum aestivum L.*) cv. Sicco as standard (Appendix 1).

2.2.3 Germinability.

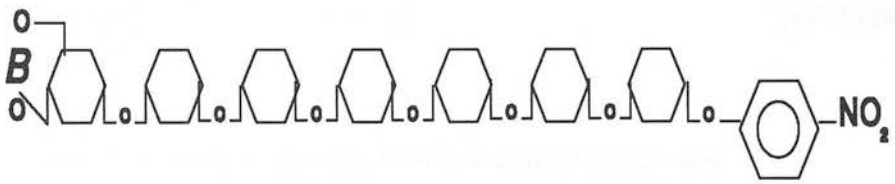
Ten ears per plot were sampled at weekly intervals. Grains from the central portion of the ear were bulked, surface sterilised in 1% sodium hypochlorite and rinsed with sterile distilled water. 25 grains were placed on moist (5ml distilled water) filter paper (Whatman No.1) in plastic petri dishes ($10cm^2$ x 2cm). Dishes were sealed with parafilm and 3 dishes per treatment were placed in incubators at 10°C and 20°C. Positions in the incubators were changed daily. Germination counts were taken after 14 days and expressed as percentage germinated grains. (Grains showing radicle protrusion were defined as germinated). A Dormancy Index (DI) was calculated according to Strand (1989):

$$DI = \frac{(\%\ \text{dormant at } 10°C \times 2) + \%\ \text{dormant at } 20°C}{3}$$

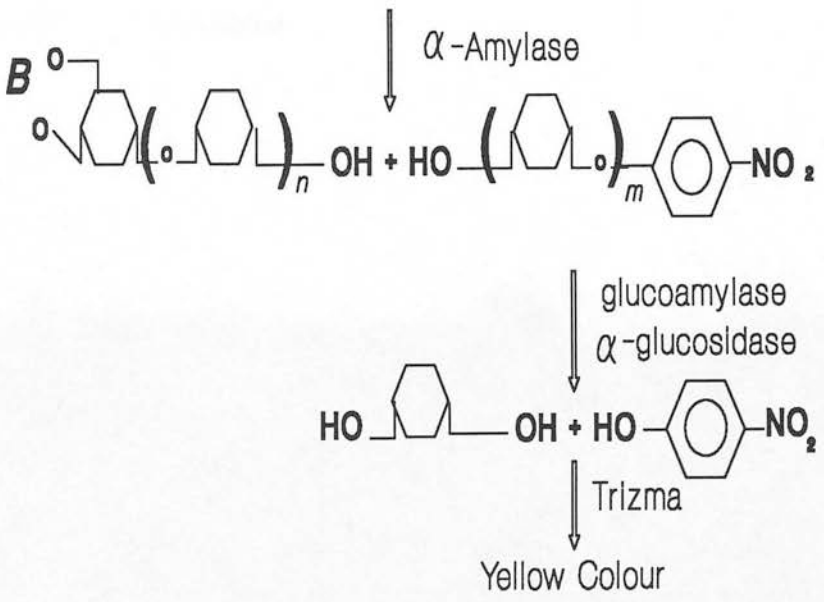
2.2.4 Alpha-amylase determination (1989)

Three replicates of ten grains from previously-frozen samples were homogenised (approx. 15s, 20,500 min^{-1}) by an Ultra Turrax T25 electrical homogeniser (Janke & Kunkel, Germany) in 7ml of buffer (sodium DL-malate (50mM), sodium chloride (50mM), calcium chloride (2mM), sodium azide (3mM); pH 5.2). Homogenisation was aided by prior sectioning of grains. The homogenate was centrifuged (Sigma 202 MK) at 4000g for ten min at 10°C. The supernatant was assayed for alpha-amylase activity using an adaptation of the method of McCleary and Sheehan (1987). The enzyme extract was pre-incubated at 40°C for 5 min to allow breakdown of partially depolymerised starch and maltosaccharides that might otherwise act as alternative substrates for alpha-amylase and lead to an underestimate of activity. Alpha-amylase substrate solution (blocked ρ-nitrophenol maltoheptaoside (545μg); glucoamylase (1U); a-glucosidase (1U)) was similarly pre-equilibrated. Enzyme extract (100μl; diluted if necessary) was then added to the substrate solution (100μl). After exactly 10 min incubation at 40°C, 1% Trizma base (1.5ml) was added, and the solution thoroughly mixed. The absorbance of the mixture at 410 nm was read within 2h by a Beckman DU62 spectrophotometer, against a distilled water blank. Extract blanks (100μl enzyme extract, 100μl malate buffer, 1.5ml Trizma) were measured for each sample. A reaction blank (100μl substrate, 100μl malate buffer, 1.5ml Trizma) and a standard (100μl enzyme extract, 100μl malate buffer, 100μl Trizma) were included in each run. The

assay is based on the following reaction (see representation below)e: Alpha-amylase attacks α-(1,4) glucosidic bonds in the blocked substrate to release a shorter length blocked maltosaccharide product and a ρ-nitrophenol substituted product. The latter is instantly cleaved to glucose and free ρ-nitrophenol by the excess quantities of glucoamylase and α–glucosidase in the substrate mixture. The free ρ-nitrophenol released by the addition of Trizma base is yellow and its absorbance at 410 nm can be measured. A standard curve using ρ-nitrophenol dissolved in Trizma base was used to calculate amount of ρ-nitrophenol in the reaction mixtures. One unit of enzyme activity is defined as the amount of enzyme which releases 1 μmole of ρ-nitrophenol/min under the defined assay conditions. Results are expressed in units per grain.



Blocked p-nitrophenyl maltoheptaoside (BPNPG7)



2.2.5 Plant Material (1990).

Four winter wheat cultivars (Apollo, Avalon, Fenman and Brock) were drilled on 10/10/89 in loam soil at Horse Park field, Bush Cereals Trials

Centre. A randomised block design, with three replicates, was used. Previous cropping was winter barley (3 years), after spring barley. Seed rate was 200 kg/ha, and P_2O_5 and K_2O (80 kg/ha each) were applied on 11/10/89. N top dressings of 50 kg/ha, 50 kg/ha and 80 kg/ha were applied on 12th March, 10th April and 26th April respectively. Weed, pest and disease control was as follows:

Herbicides	Panther	Pre-em.
Fungicides	Dorn	4/4/90
	Sportak+Dorin	26/4/90
	Sportak+Patrol	28/5/90
	Tilt Turbo+Bravo+Bavistin	18/6/90
Growth Regulator	5C Cycocel	23/4/90
Pesticide	Ambush	16/11/89

Treatments

Treatments were applied as follows, to Fenman only:

Wet: Sprayed daily (Plate 2.2) from 35 DAA until harvest with 2.2 l/m^2 of water (unlike 1989, not initially covered).

Covered: Covered by polythene tent between 35-45 DAA

Control: Untreated.

Plate 2.2 Applying water to the wetted plots.

Plots were sampled from anthesis until harvest. Separate 10-ear samples were taken from each replicate plot for measurements of fresh and dry weight, alpha-amylase and GA-sensitivity. Ears were kept in polythene bags inside an insulated cool box between field and laboratory.

2.2.6 Fresh and dry weight measurements (1990)

As for 1989, above, except that each 10-grain (1 from each ear) replicate was from an individual plot (rather than a bulked sample). In addition, ten further grains (1 from each ear) were carefully dissected into embryo and endosperm, and similar measurements made.

2.2.7 Alpha-amylase determination (1990)

As for 1989, above, except that replicates were from individual plots. Also, during the extraction procedure, samples were frozen in liquid nitrogen and then placed between cooled aluminium blocks which were hit with a mallet. This caused the grains to break up and aided homogenisation of drier, older grains.

2.2.8 Sensitivity to Gibberellic Acid (GA_3).

Five ears per plot were sampled, and grains from the outer florets of the central spikelets removed. The embryo and distal ends of the grain were removed, leaving an endosperm slice of approximately 2mm which was then surface-sterilised. For the earlier samplings this consisted of 20 min soaking in sodium hypochlorite (1% available chlorine), followed by several rinses with sterile distilled water. For older samples, with greater microbial contamination, the slices were first soaked for 2 min in 1% silver nitrate solution, followed by 2 min in 1% sodium chloride, and thorough rinsing in distilled water prior to sodium hypochlorite treatment as above. Five endosperm slices were transferred aseptically to 10ml sterile capped test tubes containing 2 ml of incubation buffer (as alpha-amylase extraction buffer, above, except sodium azide was not added) with or without 10^{-5} M GA_3. Streptomycin sulphate (50μl; 0.5mg/ml) was added to each tube. Glassware was oven sterilized and incubation buffer was filter sterilized. All work was carried out in a laminar flow cabinet. Tubes were incubated at 25°C in a shaking water bath for 72 h. Slices and incubation buffer were homogenised and centrifuged as described above, and the extract assayed for alpha-amylase activity.

2.2.9 Data analysis and presentation.

Results are described for 1989 and 1990. No statistical analysis was carried out in 1989. The plots were unreplicated and this means that an important component of variability, the plot-plot variation, cannot be estimated. As a result, there is no way of testing whether apparent differences between varieties and/or treatments are real. That is, such differences may be a reflection of differences between plots. It was considered that identification of consistent trends with time was more important than the variation associated with any individual sample point. For this reason, and for clarity, standard errors are not shown on the graphs. Coefficients of variation were, in the majority of cases, less than 10%. The alpha-amylase data for Fenman had a much higher level of variation and log transformations were carried out prior to analysis of variance. Where differences between varieties are described, this refers to the untreated controls. Most results are presented graphically. Linear regression lines were fitted to water content and percentage moisture data and used to estimate rates of water loss at different stages of grain development and ripening.

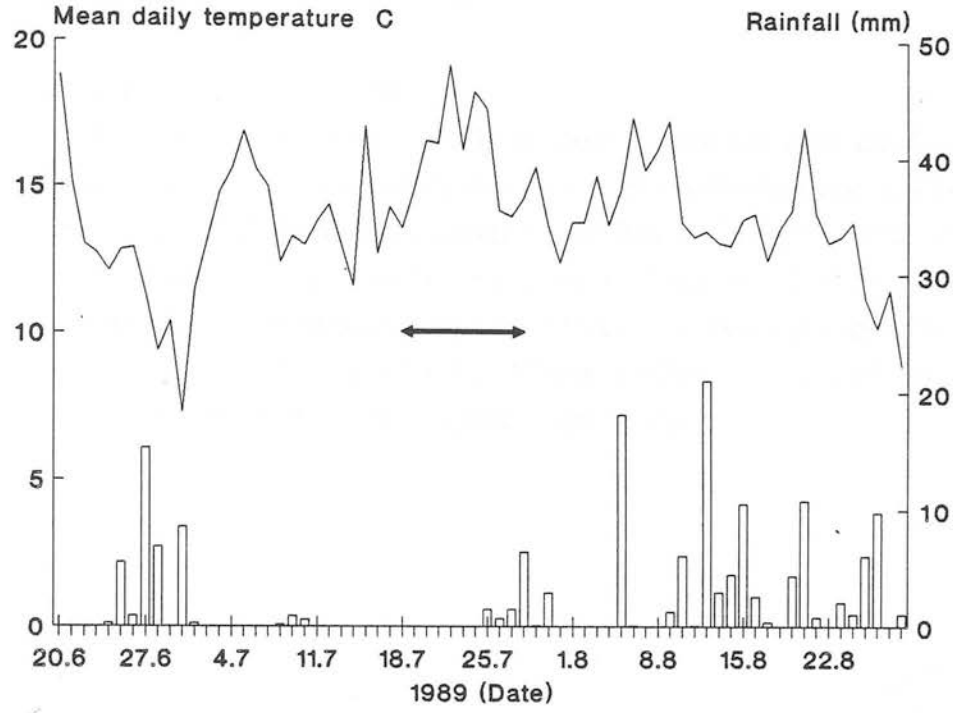
2.3 Results

2.3.1 Weather.

Mean daily temperature and rainfall for the period from anthesis until harvest are shown in Fig 2.1. Data is taken from Met. Office observations (Met. Office, 1990; 1991) at Bush House (approximately1 mile from the trial sites). Temperatures were above average in both years. July 1989 was very dry with rainfall only 19% of average. In August, rainfall was 25% above average in 1989, and 25% below average in 1990.

2.3.2 Grain development (1989).

Using the developmental scale (Appendix 1), the "ages" of the grains at 30 DAA were 35, 36, 34, and 30 "DAA" for Avalon, Fenman, Mission and Apollo respectively in 1989. This advancement of developmental age ("DAA") compared to chronological age (DAA) continued throughout the development period for Avalon and Mission but for Apollo "DAA" and DAA were in closer agreement (Fig. 2.2). Although advanced at the early stages, Fenman appeared to slow down in development towards harvest, remaining at the "dry and rubbery" (= 47 "DAA") stage. Mission and Avalon reached 51 "DAA" ("hard and floury; becoming brittle") at 44 and 47 DAA respectively while Fenman



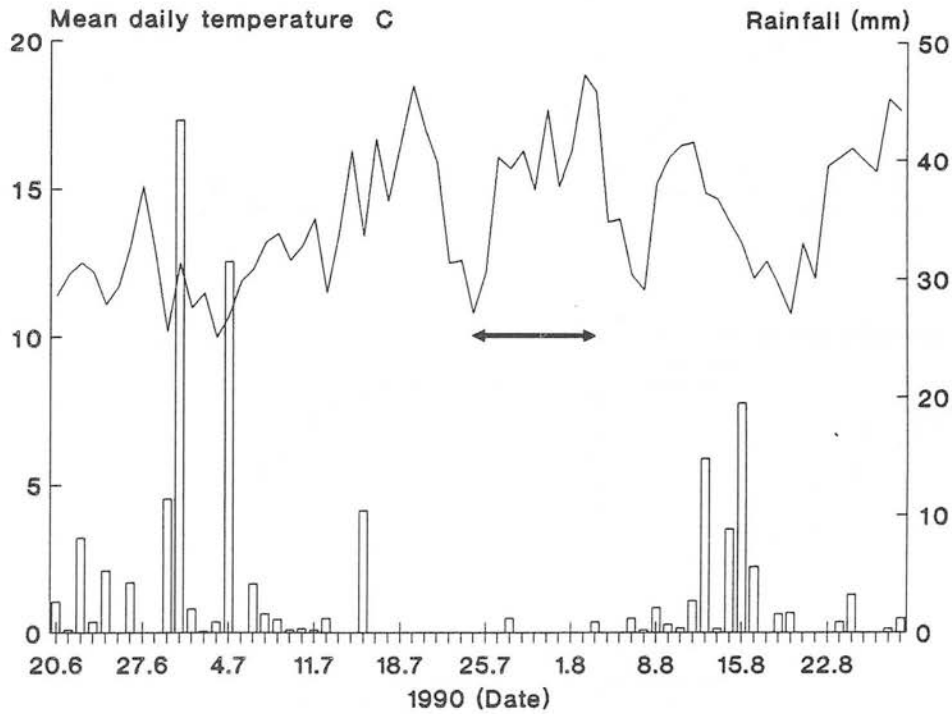


Fig. 2.1 Mean daily temperature (——) and rainfall (bars) in 1989 and 1990. ⟷ indicates duration of covering treatment.

reached it at approximately52 DAA.

2.3.3 Grain development (1990).

In 1990, "DAA" were initially in close agreement with DAA (Fig 2.2). Following this there was a twenty day period in which observed "DAA" lagged behind actual DAA for Fenman, Avalon and Brock. In Apollo, this period was of shorter duration, and development continued very much in line with actual DAA. As a comparison with 1989, at 30 DAA, the developmental "ages" were 26, 28, 29 and 31 "DAA", and 51 "DAA" was reached at 55, 58, 55 and 56 DAA for Avalon, Fenman, Brock and Apollo respectively.

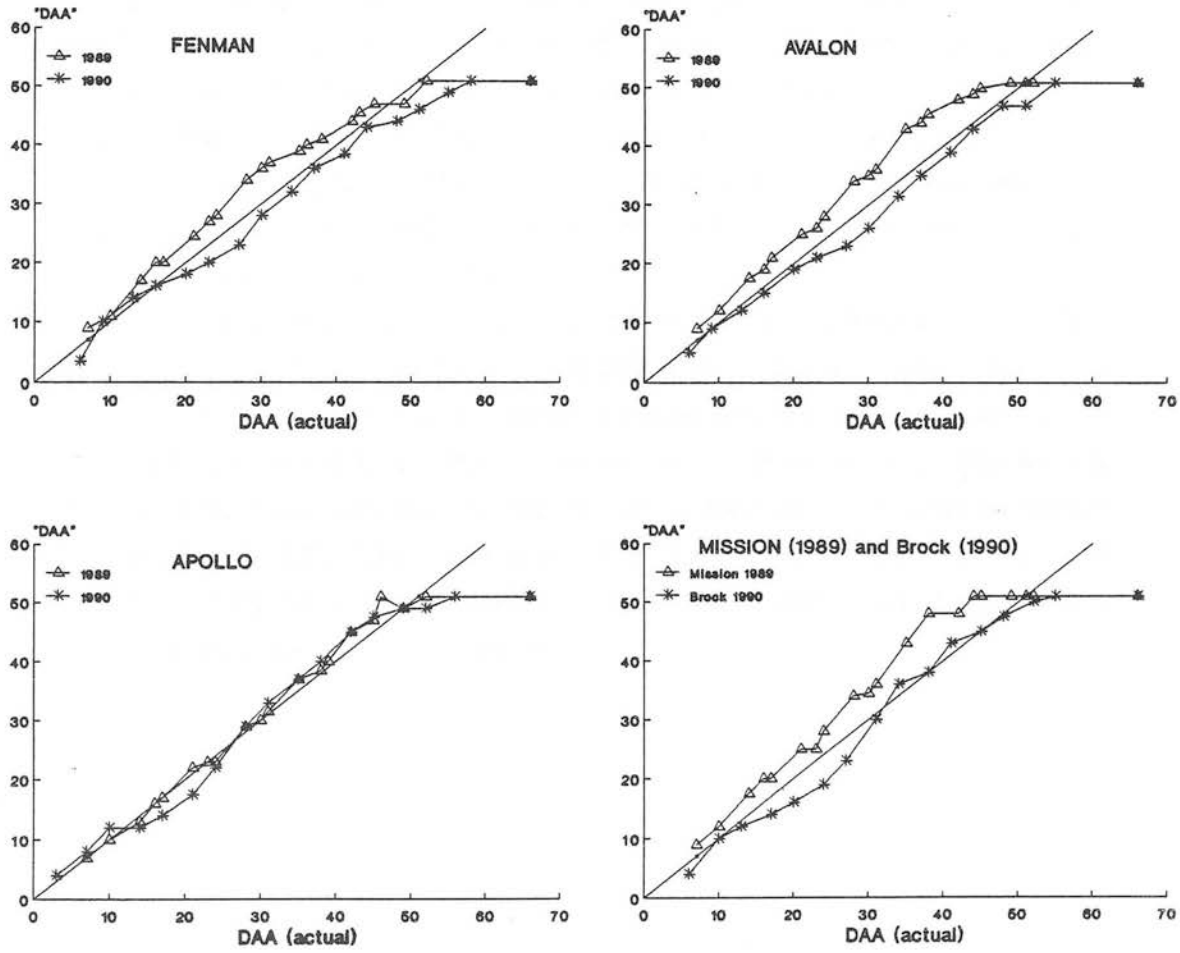


Fig. 2.2 Relationship between developmental ("DAA") and chronological (DAA) age in 1989 and 1990. Solid line (-) indicates DAA="DAA", ie. Riffkin (1987) "control".

2.3.4 Grain growth (1989).

Fresh weight of all cultivars increased to a maximum at, or just before, 30 DAA (Figs. 2.3a,b, 2.4a,b). Fresh weight then declined before reaching a constant level towards harvest. From 24 DAA onwards the fresh weight of Fenman was greater than the other three cultivars. The wetting treatment increased, and the covering treatment decreased, the fresh weight of Avalon compared to the control, from 35-49 DAA and 31-45 DAA respectively (Fig. 2.3d,f). No consistent treatment effects were observed for the other cultivars.

Dry weight rose steadily for all cultivars (Figs. 2.3a,b, 2.4a,b); maximum dry weight occurred at approximately 42 DAA (47 "DAA"), 35 DAA (43 "DAA"), 42 DAA (45 "DAA") and 35 DAA (43 "DAA") for Fenman, Avalon, Apollo and Mission respectively. Avalon and Fenman had similar rates of dry weight increase (1.75 mg/day and 1.89 mg/day respectively) until 28 DAA, after which Fenman continued to gain weight at only a slightly reduced rate until approximately 42 DAA, whereas the dry weight increase of Avalon slowed down and had levelled off by 35 DAA. A fluctuating plateau was maintained until harvest. Dry weight of the caryopses from the covered treatment was consistently lower in Avalon from 31-65 DAA; there were no consistent treatment effects for the other cultivars.

Water content shows a similar pattern in all cultivars (Figs. 2.3ab, 2.4a,b); rising to a peak at 25-28 DAA and then falling at a steady rate until about 45-50 DAA. Maximum water content was higher in Fenman, and remained at a higher level after the period of steady water loss. The wetting treatment increased, and the covered treatment decreased, the water content of Avalon (Fig. 2.3d,f). The water content of Apollo was increased between 49 and 56 DAA by the wetting treatment (Fig. 2.4c) but Mission and Fenman were unaffected by either of the treatments.

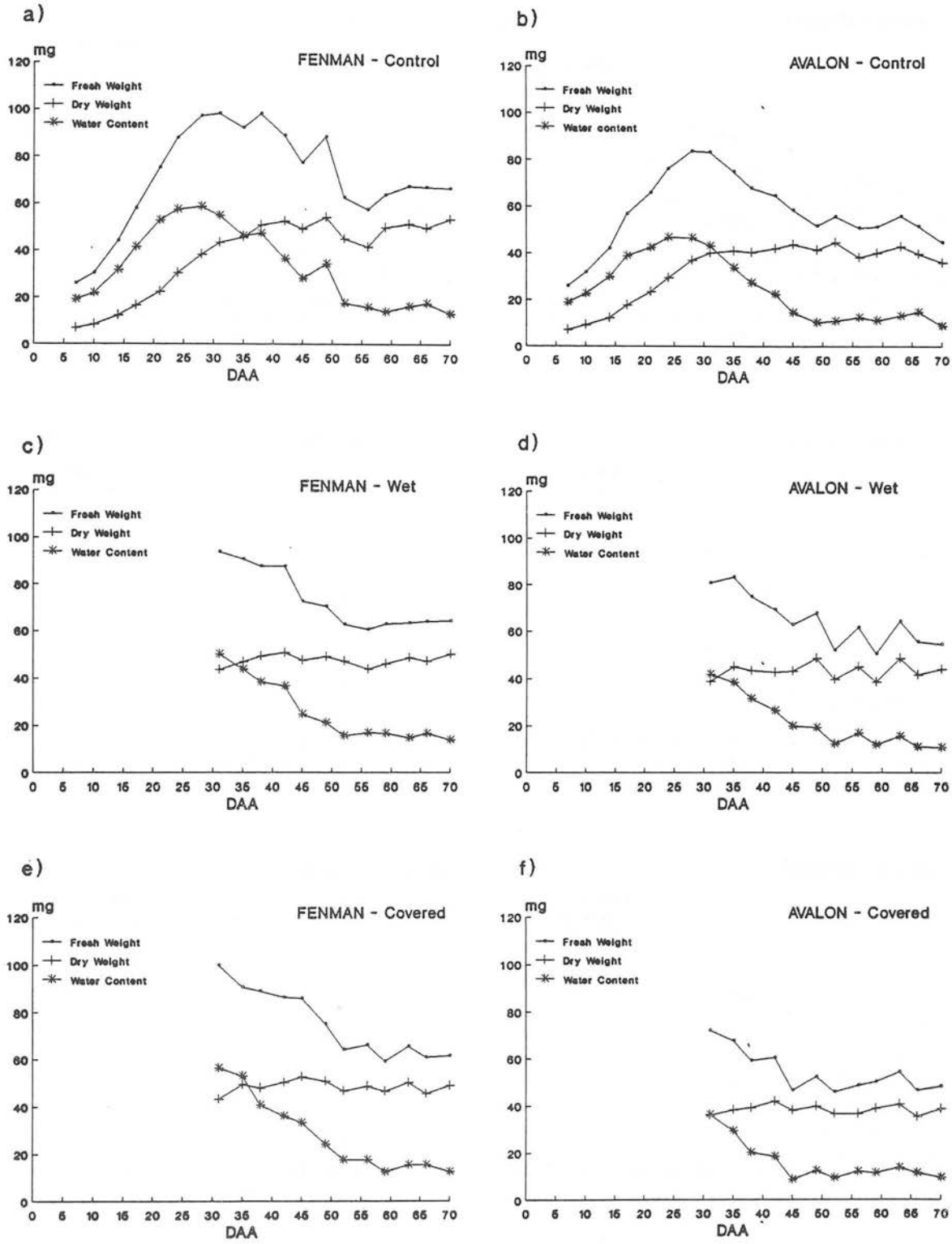


Fig. 2.3 Fresh weight, dry weight and water content of caryopses of Fenman and Avalon during grain growth and development in 1989.

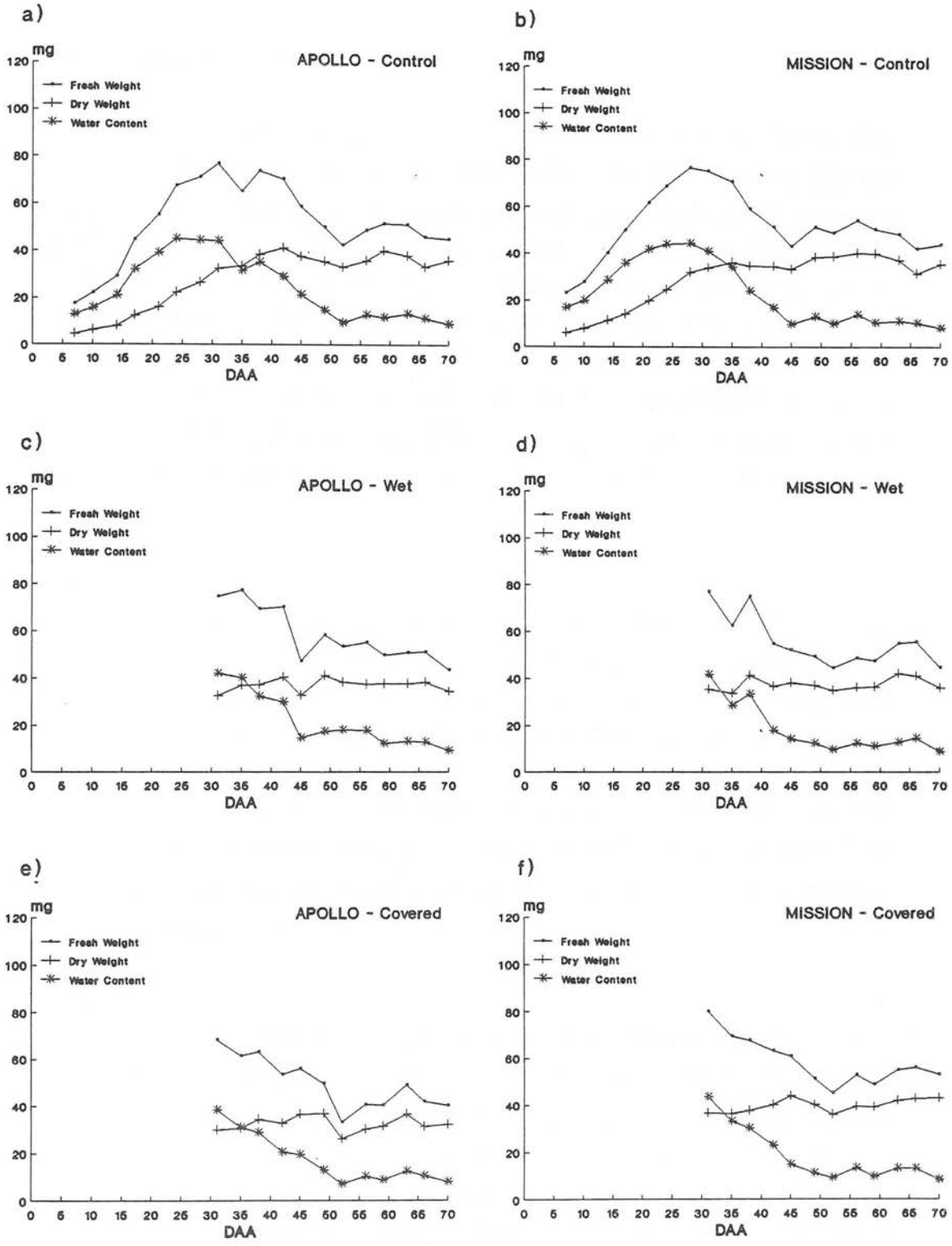


Fig. 2.4 Fresh weight, dry weight and water content of caryopses of Apollo and Mission during grain growth and development in 1989.

2.3.5 Grain growth (1990)

Caryopses

Maximum fresh weight occurred between 35 and 40 DAA (Fig. 2.5a,b,c,e). Fenman was significantly heavier than the other cultivars, but was unaffected by the treatments. Maximum dry weight was reached at 52 DAA (49 "DAA") for Brock (Fig. 2.5c), 51 DAA (47 "DAA") for Avalon (Fig. 2.5a), 52 DAA (49 "DAA") for Apollo (Fig. 2.5e) and slightly earlier, 48 DAA (44 "DAA") for Fenman (Fig. 2.5b). Treatments were applied to Fenman only, and had no effect on dry weight (Fig. 2.5b,d,f). Maximum water content occurred at about 35 DAA for Avalon and Brock (Fig 2.5a,c), about 38 for Fenman (Fig 2.5b) and 30 DAA for Apollo (Fig 2.5e). Maximum water content of Fenman was higher than the other cultivars but there was no difference between treatments.

Endosperms

Endosperms and embryos could not easily be dissected until about 23 "DAA". Below about 30% moisture (47+"DAA") clean separation was impossible. Thus, results for endosperms and embryos, start later and finish earlier than those for whole grains. Patterns of fresh and dry weight, and water content observed for endosperms (Fig 2.6a-f) were similar to those of caryopses. On a percentage basis (not shown) endosperms were generally 1-2% drier than the caryopsis as a whole. Fenman endosperms had greater maximum fresh weight, dry weight and water content than the other varieties. There were no significant treatment effects.

Embryos

In comparison to caryopses and endosperms, embryos continued to gain fresh weight, dry weight and water content until a relatively late stage of development (Fig 2.7a-f). For Fenman, there was an abrupt fall in fresh weight and water content between 58 and 62 DAA. Due to the difficulty of separation at the later stages such data is not available for Avalon, Brock and Apollo. Fenman had the largest and heaviest embryos. The treatments had no effect on fresh weight, dry weight or water content.

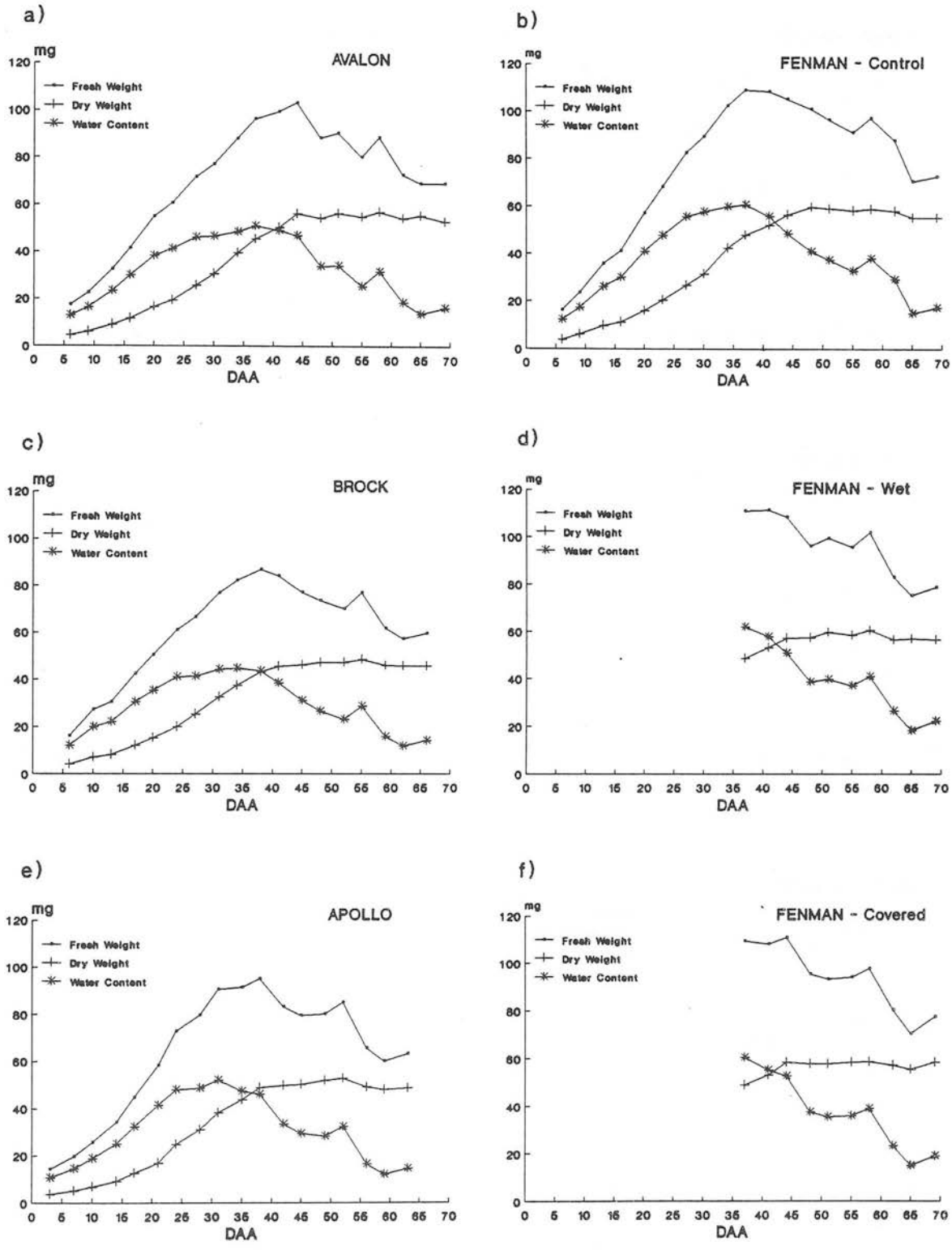


Fig. 2.5 Fresh weight, dry weight and water content of caryopses of Avalon, Fenman, Apollo and Brock during grain growth and development in 1990.

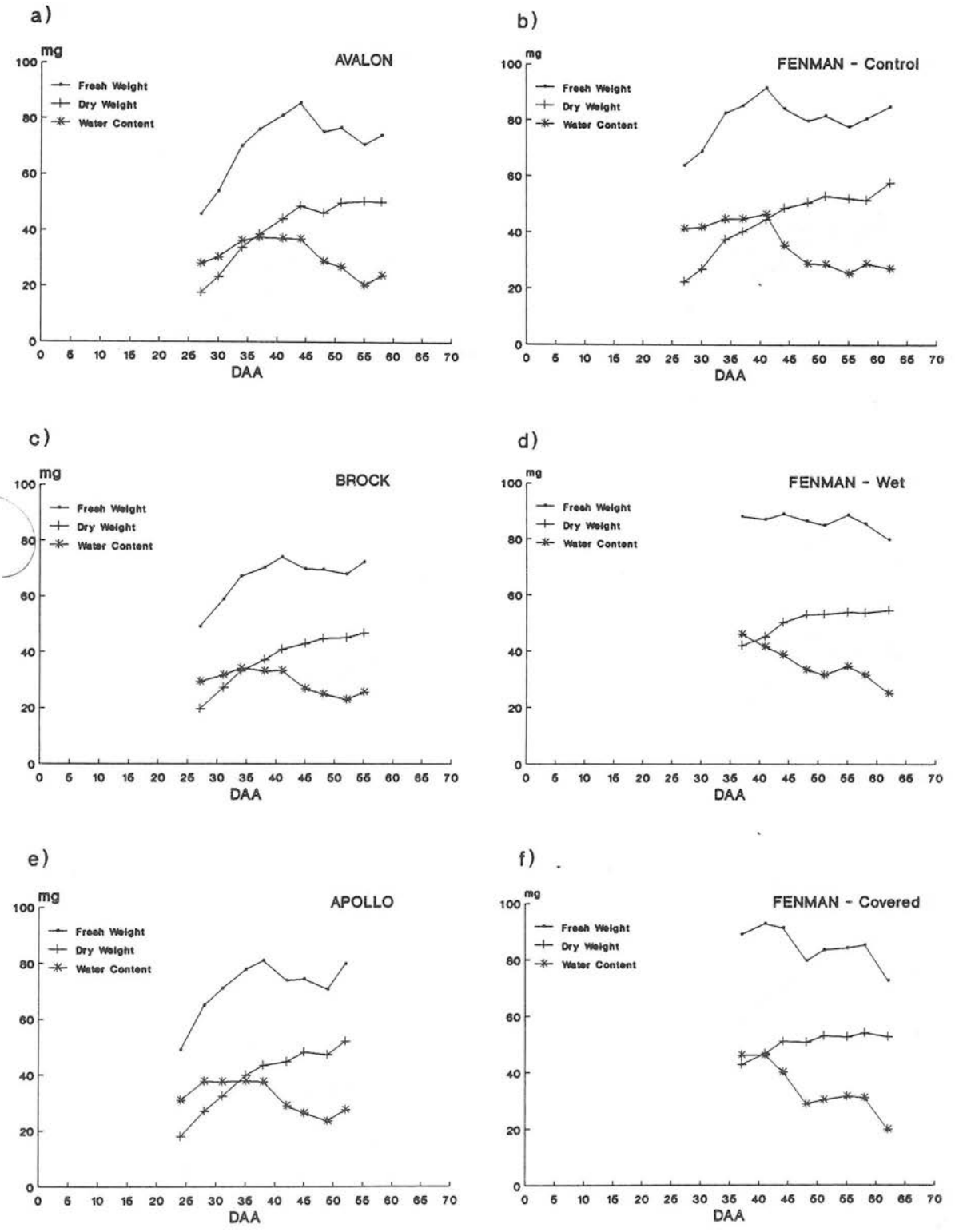


Fig. 2.6 Fresh weight, dry weight and water content of endosperms of Avalon, Fenman, Apollo and Brock during grain growth and development in 1990. Treatments applied (to Fenman only) from 36 DAA.

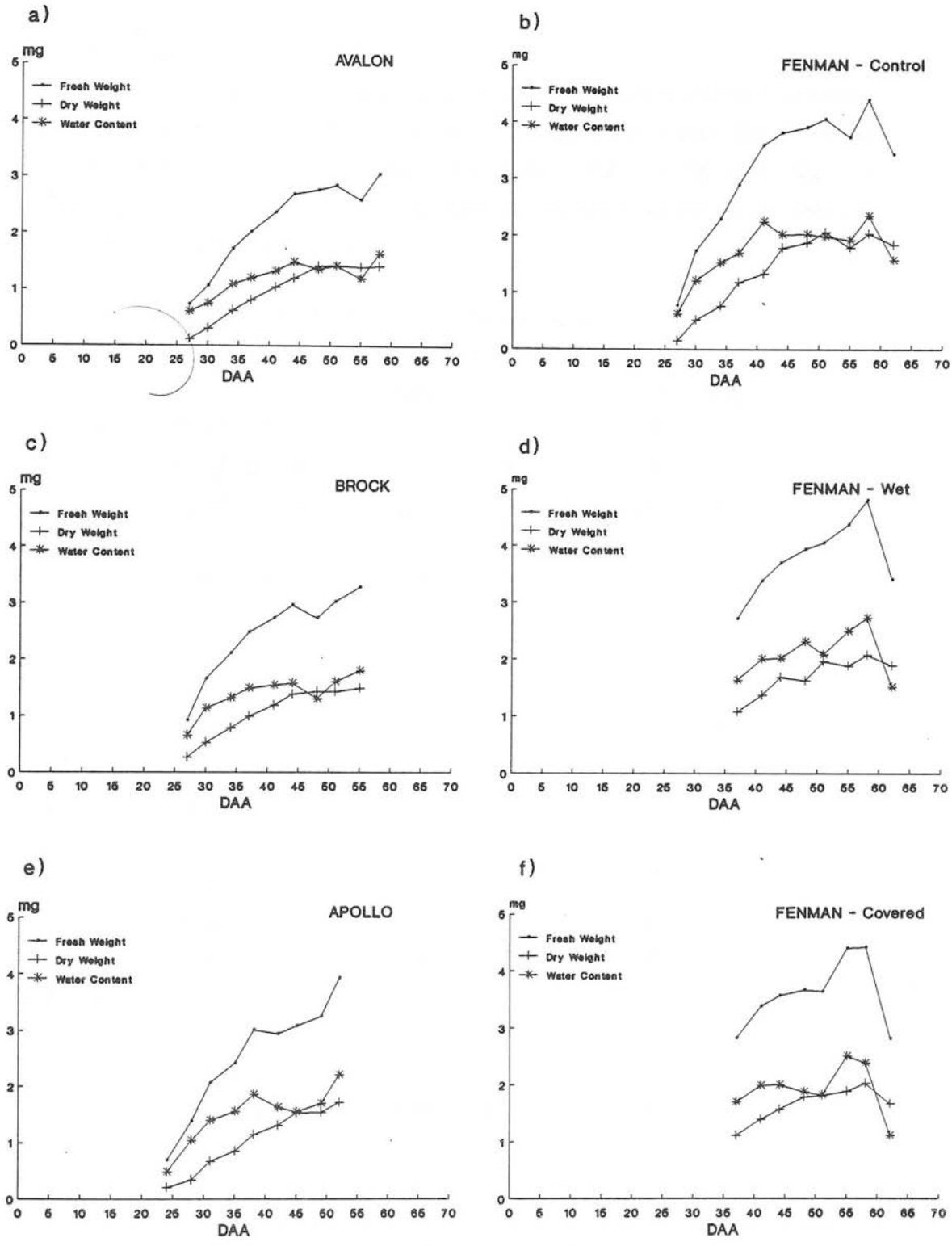


Fig. 2.7 Fresh weight, dry weight and water content of embryos of Avalon, Fenman, Apollo and Brock during grain growth and development in 1990. Treatments applied (to Fenman only) from 36 DAA.

2.3.6 Drying Rate.

Rate of water loss was estimated by linear regression of water content (absolute and percentage) data on DAA. The period of rapid water loss, (ie. from the start of water loss until the point when rain was clearly affecting the measurements), was almost linear. Results are expressed as mg water loss/day and as % water loss/day (Table 2.1).

	Duration (DAA)		Drying Rate			
			mg/day		%/day	
FENMAN	1989	1990	1989	1990	1989	1990
Control	31-45	37-48	1.81	1.84	1.38	1.41
Wet	31-45	37-48	1.63	2.15	1.27	1.47
Tent	31-45	37-48	1.81	2.0	1.31	1.46
AVALON						
Control	31-45	44-55	1.96	1.83	1.83	1.2
Wet	31-45		1.59		1.38	
Tent	31-45		1.88		2.17	
APOLLO						
Control	31-45	38-49	1.39	1.62	1.43	1.22
Wet	31-45		1.84		1.72	
Tent	31-45		1.39		1.57	
MISSION						
Control	31-45		2.28		2.27	
Wet	31-45		1.91		1.95	
Tent	31-45		1.82		1.85	
BROCK						
Control		38-52		1.52		1.27

Table 2.1 Estimated grain drying rate during the linear period of water loss.

Percentage moisture gave a slightly better fit than did water content data; between 85% (Apollo, 1990) and 99% (Apollo-Covered,1989) of the variation in percentage moisture was explained by the fitted equation. The duration of the initial water loss period was fairly similar between cultivars; in 1990 however, water loss began slightly later and the duration was shorter. The duration is difficult to estimate accurately and is somewhat dependent on rainfall. In 1989, Mission had the fastest rate of water loss (%/day), and

Fenman the slowest. In 1990, however, Fenman had the fastest rate and Avalon, Apollo and Brock all had lower rates. Fenman had a slightly faster rate of water loss (%/day) in 1990 compared to 1989, whereas Avalon and Apollo both had lower rates in 1990. The treatments did not have a consistent effect on the different cultivars. The wetted treatment had the slowest, and the covered, the fastest, drying rate (%/day) in Avalon. In Apollo however, the wetted treatment had the fastest drying rate. In Fenman, there was little effect of the treatments in either year.

	1989 %/day	1990 %/day
FENMAN		
Control	1.09	0.942
Wet	0.91	0.847
Tent	1.18	0.945
AVALON		
Control	1.94	1.08
Wet	1.34	
Tent	2.27	
APOLLO		
Control	1.87	1.1
Wet	0.898	
Tent	1.75	
MISSION		
Control	2.6	
Wet	1.54	
Tent	2.13	
BROCK		
Control		1.04

Table 2.2 Estimated grain drying rates, over the period from 40-23% moisture, in 1989 and 1990.

Grain drying rate between approximately 40%-23% moisture was also estimated using linear regression (Table 2.2). The regression equations were generally of much poorer fit than during the initial stage of water loss, only explaining 59% of the variation in percentage moisture of Fenman-Covered in

1990 for example. During this period, in 1989 and 1990, Fenman had the slowest drying rate. Within varieties, the wetting treatment always had the slowest drying rate. The covered treatment produced the fastest drying rate in both Avalon and Fenman, but the difference between treatments was greater in Avalon. Drying rates of endosperms were also calculated in 1990 (not shown), and a similar pattern was found, with Fenman having the slowest rate of drying.

2.3.7 Germinability and Dormancy (1989)

Onset of whole-grain germinability (Fig 2.8) occurred between 22 and 30 DAA. For all cultivars except Apollo, acquisition of germinability was followed by a rapid rise in percentage germination at 10°C. Apollo showed a much slower increase and attained maximum germination at 60 DAA compared to 35 DAA for the other cultivars. Response to germination temperature varied between cultivars. Fenman and Apollo had increased germination at 10°C compared to 20°C, but the difference was not great, and the pattern of germinability with time was similar. For Avalon and Mission, the cooler temperature caused a relatively larger increase in germination. Treatment effects were rarely consistent. For Apollo both the wet and covered treatments had a higher germination percentage than the control at 10°C, but not at 20°C. Wetting lowered the germination percentage of Mission at 20°C, and Avalon at 10°C.

Avalon had the lowest dormancy index (DI) and Apollo the highest, for much of the period from 25 DAA (Fig. 2.9). Wetting and covering reduced the DI in Apollo, but wetting increased DI in Avalon. It is difficult to discern treatment effects on Mission, but the wetted sample had a higher DI at 36 DAA. The DI of Fenman was unaffected by either treatment.

2.3.8 Alpha-amylase

Activity of extractable alpha-amylase of intact caryopses during grain development and maturation is shown in Figs. 2.10 (Avalon, Apollo, Mission and Brock) and 2.11 (Fenman; note log scale). All cultivars had similar levels during early grain development in 1989 (alpha-amylase activity over this period was not assayed in 1990). Alpha-amylase levels in Avalon, Apollo and Mission then declined from approximately 15 DAA until 40 DAA and remained at this low level until 70 DAA (Figs 2.10a,c,e). There were no treatment effects on these three cultivars. In 1990, a similar pattern of declining alpha-amylase was recorded for Avalon, Apollo and Brock(Figs 2.10b,d,f). In Fenman, in 1989,

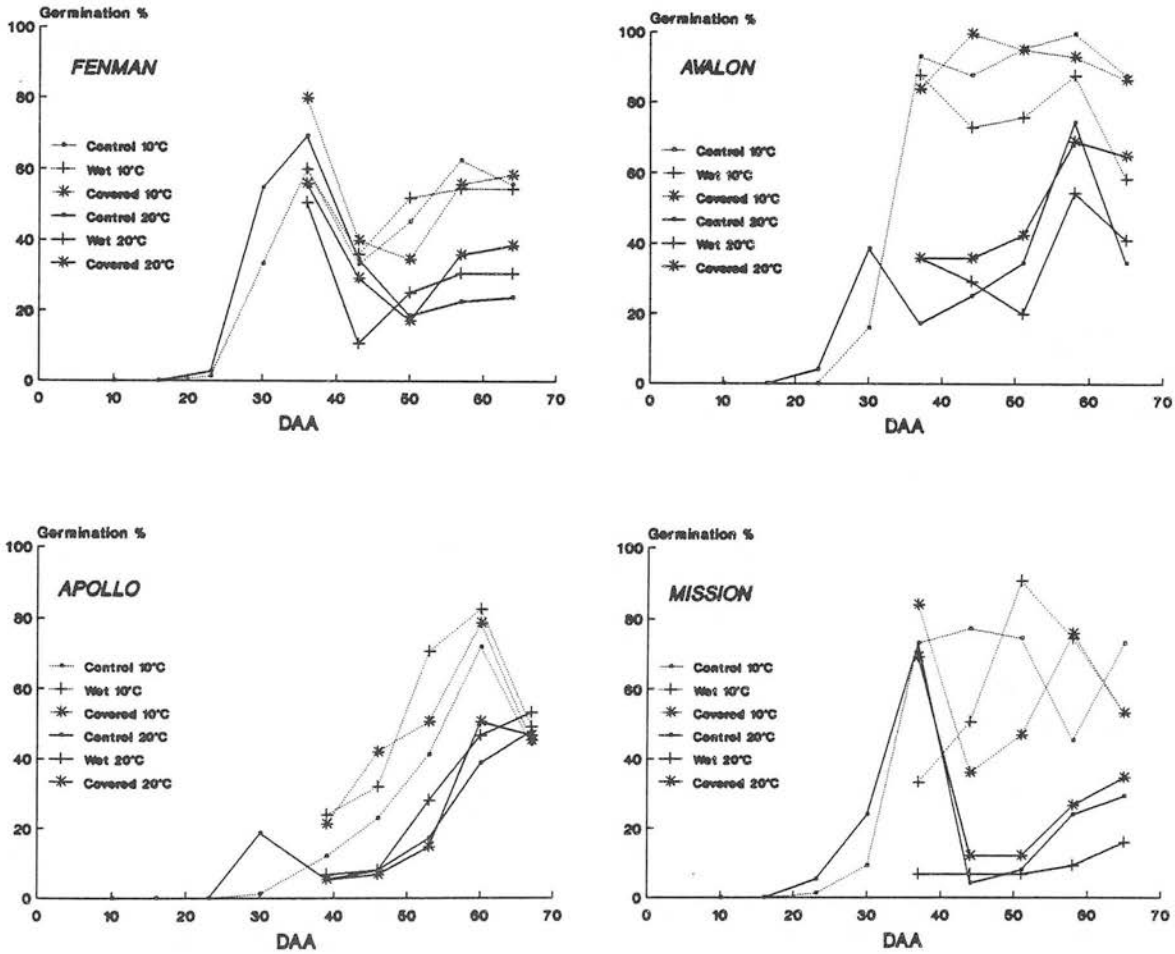


Fig 2.8 Germination of Fenman, Avalon, Apollo and Mission at 10°C (....) and 20°C (——) in 1989.

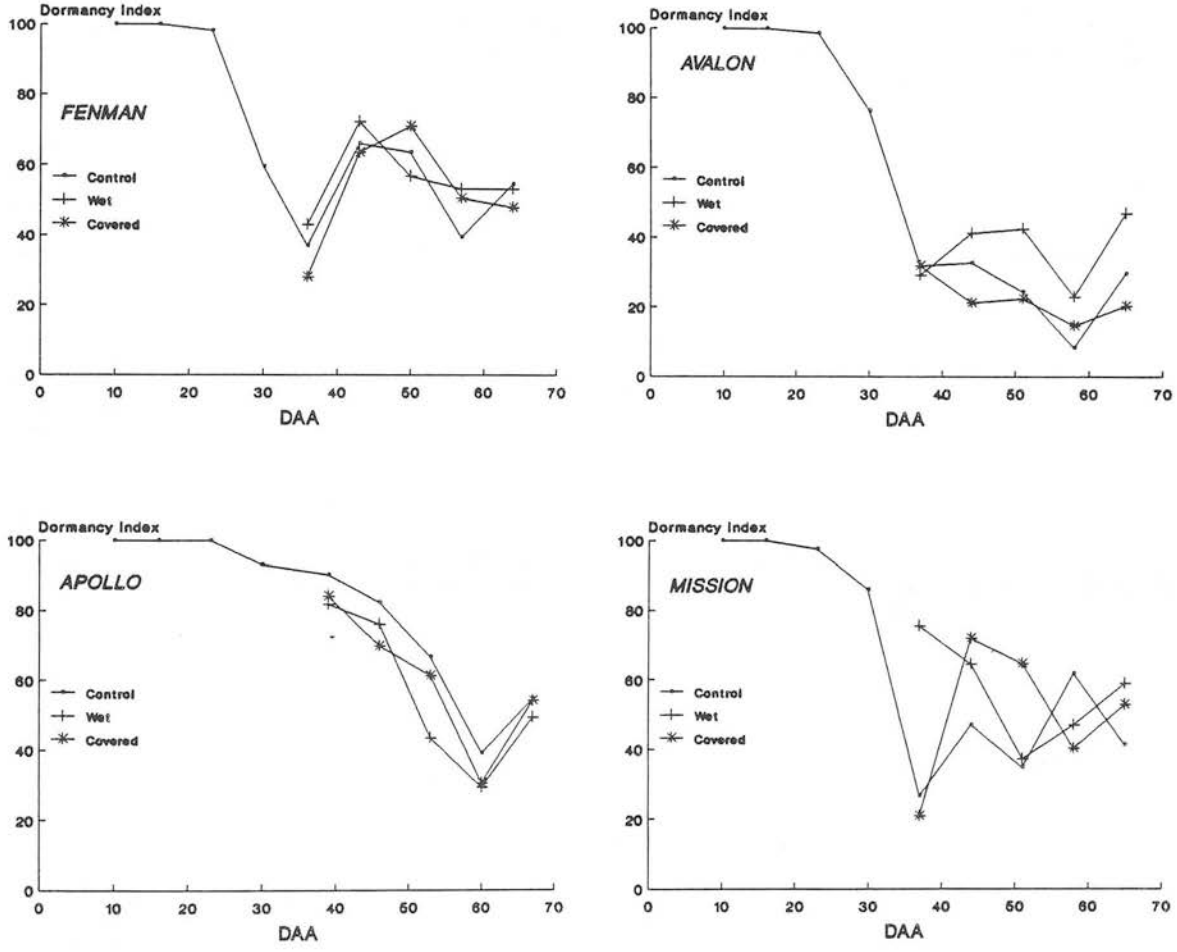


Fig. 2.9 Dormancy Index of Fenman, Avalon, Apollo and Mission in 1989

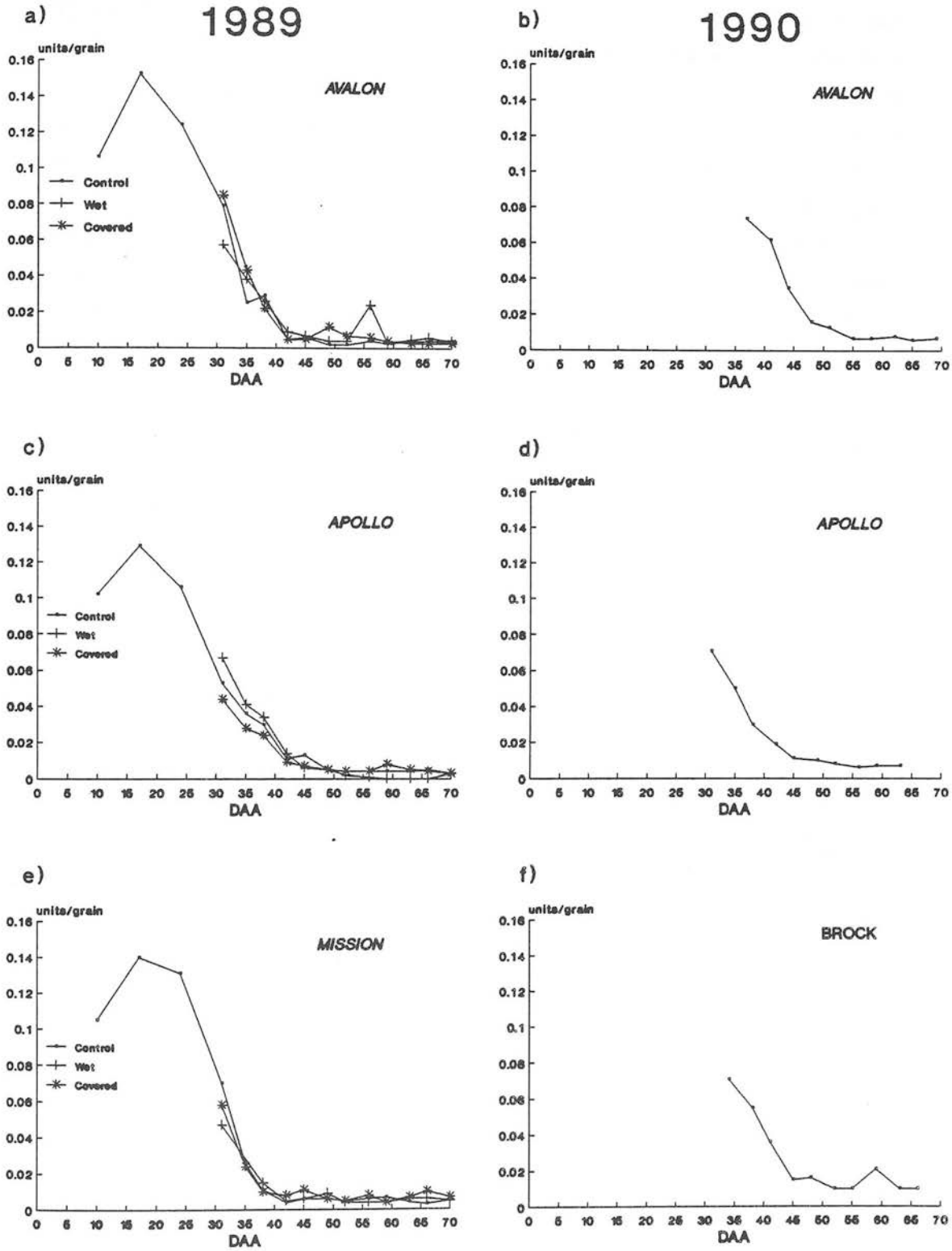


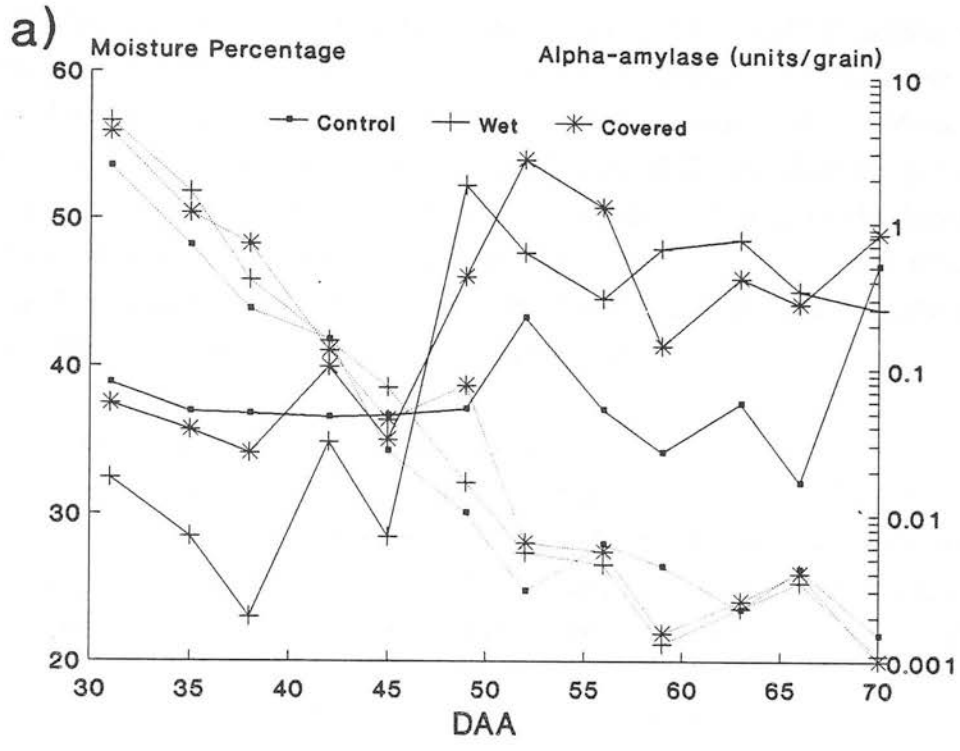
Fig 2.10 Alpha-amylase activity of caryopses in 1989 and 1990.

levels of alpha-amylase were similar to those of Avalon, Apollo and Mission until 30 DAA (data not shown). From 35 DAA the alpha-amylase activity of the control and the treated caryopses of Fenman was higher than that of Avalon, Apollo or Mission. From 30 until 45 DAA the caryopses from the wetted treatment of Fenman had lower levels of alpha-amylase than the control or the covered (Fig 2.11a) ; activity then rose rapidly in both treatments and remained higher than the control until harvest, by which time the activity of the control samples had risen to a similar level. The covered treatment had higher alpha-amylase activity than the control or the wetted treatment at 52, 56 and 70 DAA.

DAA		>0.1 units/grain	
		No. grains	units/grain
49	Control	0	-
	Wet	16	3.685
	Covered	17	0.924
52	Control	3	2.388
	Wet	13	3.467
	Covered	12	1.763
56	Control	4	0.782
	Wet	1	2.665
	Covered	13	4.236
59	Control	0	-
	Wet	1	6.36
	Covered	4	2.89
63	Control	2	6.465
	Wet	13	3.662
	Covered	14	3.273
66	Control	0	-
	Wet	6	4.219
	Covered	16	2.515

Table 2.3 Effect of wetting and covering treatments on number of grains with alpha-amylase activity greater than 0.1 units (1989)

Three grains from each of the sampled ears of Fenman were later assayed for extractable alpha-amylase activity. Grains with high activity were often located in the same ear. Table 2.3 summarises the data (the original data are shown in



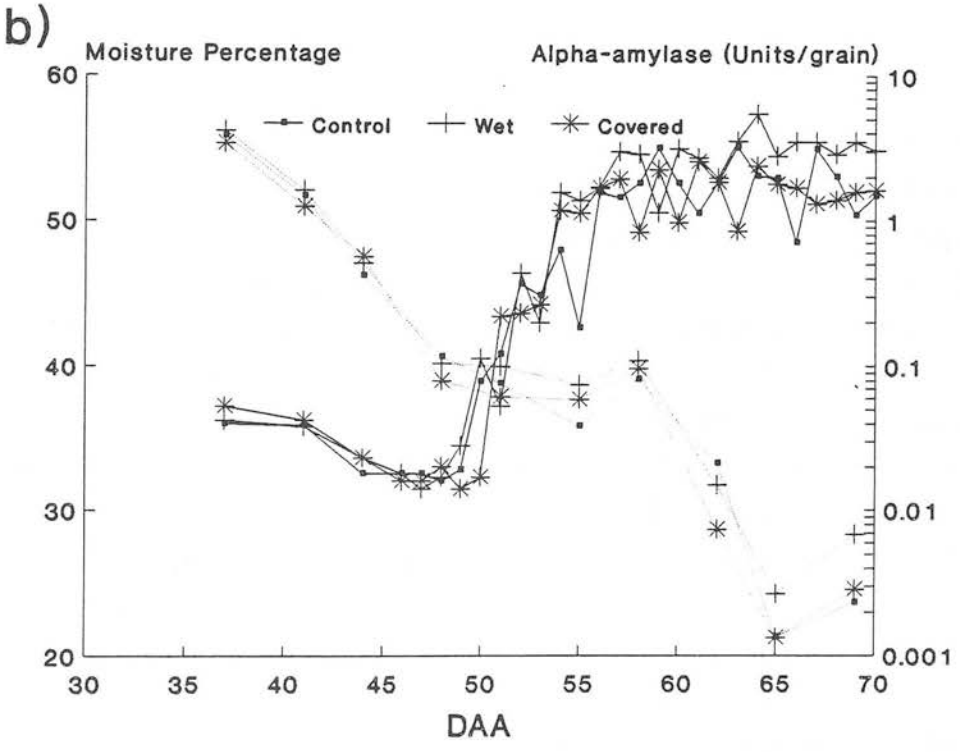


Fig 2.11 Alpha-amylase activity (——) and moisture (....) percentage of Fenman in 1989 and 1990.

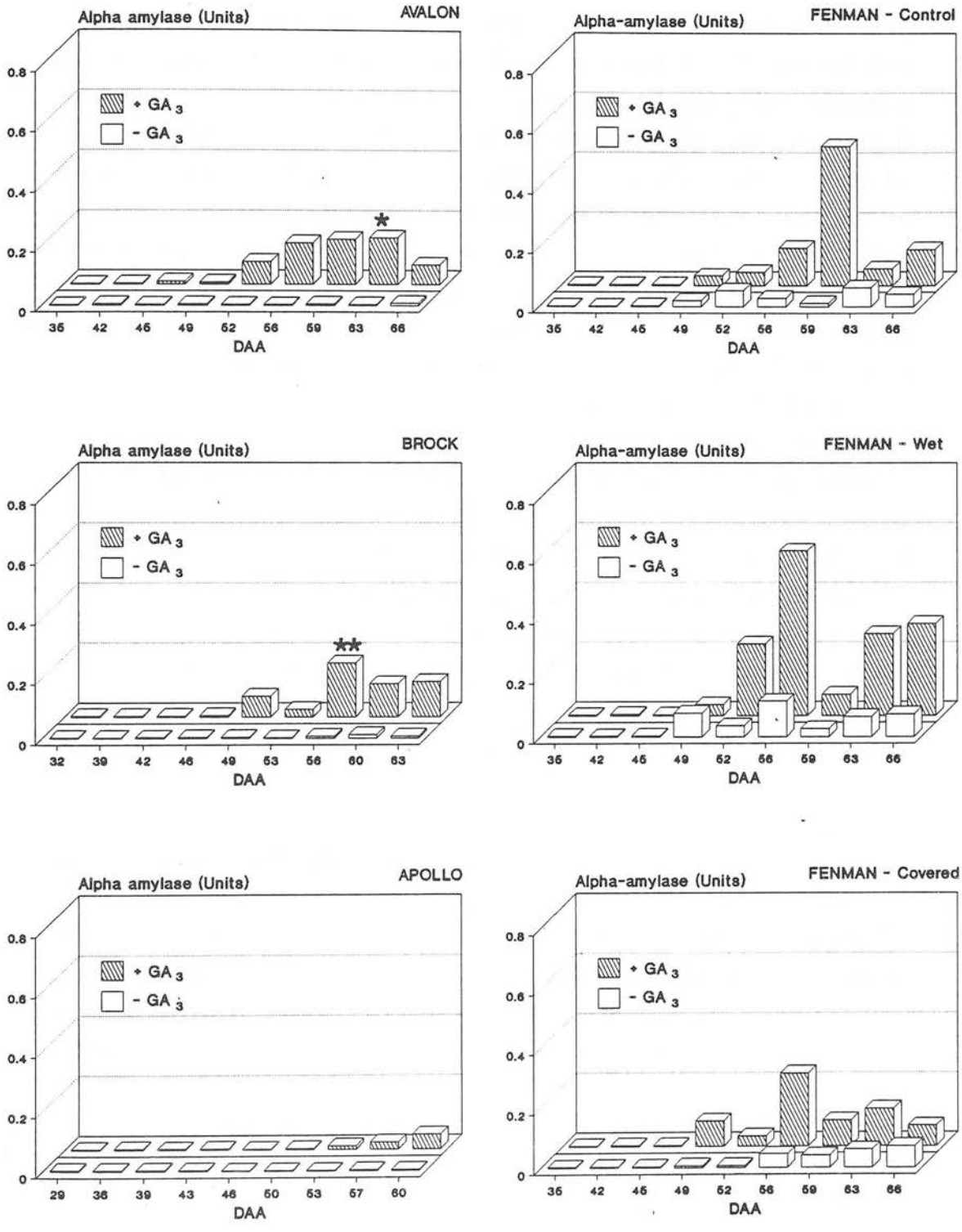
appendix 2). In the control, there were fewer grains with high activity (> 0.1 units). In 1990, there were no clear treatment effects on extractable alpha-amylase activity in Fenman (Fig 2.11b). Alpha-amylase activity of caryopses from all treatments rose rapidly between 48 and 57 DAA. Moisture percentage is also shown in Fig 2.11. In 1989, at the time (45 DAA) of the increase in alpha-amylase activity moisture percentage of the wet and covered treatments was 34% and 38% respectively. In 1990, the increase in alpha-amylase activity occurred at about 40% moisture in all treatments.

2.3.9 Sensitivity to Gibberellic Acid (GA_3).

The response of de-embryonated endosperm slices to GA_3 is shown in Fig. 2.12. Alpha-amylase activity in the GA_3-incubated treatments was first recorded at approximately 49 DAA (Fenman and Brock), 52 DAA (Avalon), and 53 DAA (Apollo). "DAA" and moisture percentage at the onset of GA_3-sensitivity was as follows:

	DAA	"DAA"	% moisture
Fenman	49	44	40
Avalon	52	47	36
Apollo	53	47+	36
Brock	49	47	35

The onset of GA_3-sensitivity in Fenman occurred at an earlier "age" and higher moisture content. With the exception of Fenman-Control at 52 and 63 DAA, Fenman-Wet at 49 DAA, and Fenman-Covered at 66 DAA, the mean alpha-amylase activity was higher in the GA_3-incubated endosperm slices than in slices incubated without GA_3. However, the difference between plus and minus GA_3 is only significant on two occasions (Avalon, 63 DAA and Brock, 56 DAA). This is mainly due to the fact that the amount of alpha-amylase produced in the presence of GA_3 was not uniform between the three replicates. With Avalon, for instance, the mean response to GA_3 was very similar at 59 and 63 DAA, but the responses of individual replicates were 0.137, 0.27, 0.035, and 0.127, 0.193, 0.138 at 59 and 63 DAA respectively; the difference was significant at 63 but not at 59 DAA. Contributing to the lack of significance in Fenman was the fact that alpha-amylase was also present in most replicates, but at varying levels, in the absence of GA_3. Levels of alpha-amylase in the GA_3-incubated treatments were lowest for Apollo. The wetted treatment of Fenman had higher levels of alpha-amylase in the presence of GA_3, than the control or the covered treatment, except at 59 DAA.



2.12 Alpha-amylase activity in endosperm slices incubated with or without GA_3. * and ** indicate significance at 0.05 and 0.01 respectively.

2.4 Discussion

In presenting the data, consideration was given to the question of the most appropriate scale for the X axis. Date is perhaps the simplest, but does not take into account differences in maturity due to genotype (when comparing varieties in the same year) or environment (when comparing the same variety in different years). DAA attempts to overcome the former but does not allow for differential rates of development after anthesis. Degree Days after Anthesis corrects for differences in time to reach anthesis, and, by including a temperature sum, takes account of some of the environmental effects; both year to year differences, and also the different environments experienced by genotypes at the same physiological (but not chronological) stages of development. "DAA", should encompass all of the variation due to environment and, as such, should give good discrimination between varieties. However the scale does not cover the last part of the grain drying period, when significant biochemical activity may still be occurring. The use of moisture percentage as an X axis scale produces a pattern similar to "DAA", and continues until harvest. However, moisture percentage may rise and fall over the last stage of grain drying and cannot therefore give a true indication of time. Thus, there is no one scale that produces an "ideal" graph. It was decided to use DAA because, at present, it is the mostly widely used in published data and comparisons are therefore possible (bearing in mind probable environmental effects on rate of development).

2.4.1 Fresh and Dry Weight Changes

Based on observations on June 14th and 21st, the date of anthesis in 1989 was estimated as 19th June. More frequent observations in 1990 provided an accurate date of anthesis for all cultivars. Date of anthesis was defined as the date at which approximately 75% of the ears had anthesed, and occurred on 19th June (Fenman and Avalon), 22nd June (Brock) and 25th June (Apollo). The peak in fresh weight at 30 DAA in 1989 was about 7 days earlier than Riffkin (1987) observed in 1983, the year in which his developmental aging scale was produced. Actual days (DAA) lagged behind developmental "days" throughout grain development; ie. development was faster than the Riffkin (1987) "control". This reflects the generally warm and dry weather in June and July 1989, when mean daily temperature and sunshine were above average (Meterological Office, 1989). In 1990, maximum fresh weight was reached at approximately the same DAA as Riffkin recorded, and chronological age and

developmental "age" were in much closer agreement throughout grain development. However, temperature and sunshine data were above average in 1990 also, a specific "day degree" being reached only aproximately one day earlier in 1989 compared to 1990. The explanation for the slower rate of grain development in 1990 must lie in some other factor. Rainfall was greater during the early stages of grain development in 1990. Neither treatment resulted in alteration of the rate of grain development; this is not surprising, given an estimated error of ±2 days in the age scale (Riffkin, 1987), and variability within plots (discussed below).

Maximum dry weight did not occur at the same "DAA" for all cultivars or for a particular cultivar in different years. Riffkin (1987) found that morphological events were closely related to gross changes in fresh weight, dry weight and water content. His data were derived from one cultivar, Sicco, and the present data suggest that cultivars may differ in the extent to which morphological development coincides with particular stages of grain growth. The unevenness of the plateau after the decline in fresh weight in 1989 may represent increased moisture due to rainfall, but the fact that dry weight measurements show a similar fluctuation suggests that it is at least partly due to sampling variation. (Calculation of 95% confidence intervals for sequential samples shows that their ranges overlap.) This is borne out by the 1990 data, in which similar fluctuations in fresh weight are apparent, while the dry weight curves are much smoother. The amount of plant material from which a sample could be drawn was much smaller in 1989, the plots being unreplicated and divided into thirds by the treatments. Towards the end of the experiment, previous sampling had left the plots rather sparse, possibly contributing to the sampling of unrepresentative ears. An increasing variability in physiological "age", possibly due to uneven ripening, was noted at the later stages in both years. This may have contributed to the variation associated with fresh weight and water content measurements, but should not have affected dry weight because it occurred after maximum dry weight was recorded. Fluctuations in dry weight throughout grain development have been reported by Jenkins *et al.* (1974). They correlated the changes with similar fluctuations in pericarp alpha-amylase, and suggested that starch turnover was occurring in the pericarp. While this may explain fluctuations at an early stage of development it does not explain their later data, or the present data, for which sampling error remains the most likely explanation.

The heavier grain dry weight of Fenman has been noted previously by

Caley (1986) in her comparison of starch deposition in the varieties Fenman and Broom. She found that the increased dry weight was partly due to greater amounts of starch deposited in the endosperm, and, that while the duration of grain filling was shorter in Fenman, the rate of starch deposition was greater. Starch deposition rate was not studied in the present work, but the almost linear period of dry weight increase was of longer duration in Fenman compared to the other cultivars.

2.4.2 Germinability and Dormancy

Dormancy is the major factor involved in resistance to PHS. What is not known is how it affects PMAA. However, identification of cultivars that are resistant to PHS but contain high levels of alpha-amylase (Mares, 1987a) suggest that there may not be a relationship between dormancy and PMAA. Examination of Fig 2.3, 2.4 and 2.8 shows that the first appearance of germinability was at approximately the same time as maximum water content. This is in agreement with Mitchell *et al.* (1980). All cultivars had higher germination at 10°C than 20°C; an indication of temperature-sensitive dormancy. Apart from this, the cultivars varied considerably in their germination response. The data for germination of Fenman and Mission at 20°C show a similar pattern to that recorded by Mitchell *et al.* (1980) for field-grown wheat in 1976; i.e. a peak, followed by a reduction (presumably due to dormancy), and then an increase towards harvest, as dormancy is lost. With Avalon, the peak occurred at a later stage and with Apollo it appears that germination is still increasing even at the last sample date. However, it is possible that there is confusion between dormancy and immaturity. The high level of germination observed at 10°C from 36 DAA onwards for Avalon indicates that the lower level at 20°C was due to temperature-sensitive dormancy. This was gradually lost, before increasing again after 58 DAA. The reason for this late increase is unknown; (secondary dormancy has been reported from field trials, but under rather cooler temperatures and higher humidity (Belderok and Habekotté, 1980). In Apollo, by comparison, the difference between the two germination temperatures is not as great, suggesting lower dormancy. Secondly, the pattern of increasing germinability is similar at both temperatures, suggesting a gradual acquisition of germinability. Cultivars are known to differ in the rate at which they mature, depending on which "maturity trait" is examined (Gordon *et al.*, 1979). In this case, it appears that germinability is a relatively late occurrence in Apollo. Alternatively, it may be

that another type of dormancy, insensitive to temperature, is present, and that the gradual increase in germination represents a loss of dormancy rather than an increase in maturity. The effect of the wetting treatment is particularly apparent for Mission at 37 DAA and for Avalon, over the period from 43-66 DAA. It was suggested (Mitchell *et al.*, 1980) that drying of the pericarp is critical to the onset of germinability, and it may be that while the treatment did not significantly affect whole-grain moisture it was enough to delay drying of the pericarp. It is of interest that Mission and Avalon appeared most sensitive to germination temperature and it may be that there are genotypic differences in environmental sensitivity. The objective of examining germinability during grain development was to obtain information on the dormancy characteristics of the individual cultivars and thus to determine whether alpha-amylase assayed was due to PHS or PMAA. In the absence of PHS and PMAA in Avalon, Mission and Apollo, the significance of the results is unclear. Avalon had a fairly low level of dormancy at harvest and would therefore have been at risk of sprouting had weather conditions been appropriate. This corresponds with its lower "resistance to sprouting" rating in NIAB lists (Mann, 1980). Fenman was the only cultivar in which significant levels of alpha-amylase were recorded. No visible sprouting was observed, and the Dormancy Index indicates that a high level of dormancy was still present at harvest. Therefore it seems fair to conclude that the alpha-amylase present was due to PMAA.

2.4.3 Sensitivity to Gibberellic Acid.

It is often assumed that, since GA appears to regulate alpha-amylase synthesis in "normally"-germinating grains, it may play a similar role in the control of alpha-amylase production in non-sprouted grain. Gale *et al.* (1983), in their model of the effect of drying rate on alpha-amylase activity, assumed that both aleurone and scutellum were sensitive to GA. In support of the involvement of GA they cited the fact that the alpha-amylase isozymes produced during PMAA are the same as those produced during germination, and also that the only genotypes in which premature alpha-amylase production was not observed were those containing the GA-insensitive gene *Rht 3*. Cornford *et al.*, (1987) also propose that the development of aleurone responsiveness to GA_3 is a requirement for the high alpha-amylase production observed in Fenman. Developing grain is normally insensitive to GA_3 until about the time of grain water loss (King, 1976, Armstrong *et al.*, 1982), but it has been shown that aleurone sensitivity can be induced in younger grains by

detaching and slow drying (Nicholls, 1979), by pre-incubation (Norman *et al.*, 1982, Armstrong *et al.*, 1982), by detachment and maintenance at high humidity (King and Gale, 1980, Nicholls, 1986a), and by ear culture in water, or mannitol or sorbitol-containing media (Nicholls, 1986b). The onset of prematurity alpha-amylase does not appear to occur before about 40% moisture content and therefore it is unlikely that it is due to any unusually early sensitising of immature aleurone tissue. Cornford and Black (1985) observed PMAA in Fenman, but found no response to GA_3 in 25 DAA half-grains unless they had been pre-incubated for 72 hours. Few studies have examined the timing of the normal GA-response in relation to alpha-amylase production in ripening grains of resistant and susceptible cultivars. Gale *et al.*, (1983) found a "window" of aleurone responsiveness coinciding with a period of whole-grain germinability at approximately 40-50% moisture content. This coincided approximately with the onset of alpha-amylase production. However, aleurone sensitivity was then apparently lost, although alpha-amylase activity continued to rise. In the present work, incubated endosperm slices of Fenman first showed alpha-amylase activity at 49 DAA. This coincides very well with the start of the steep rise in alpha-amylase production in whole grains. However, due to the fact that some replicates showed alpha-amylase activity in the absence of GA_3 and others had very little activity in its presence, it appears that GA-sensitivity was not uniform throughout the sample. That this variation was found at all sample dates from 49-66 DAA indicates that it was not just due to normal variation in the rate of grain development. It is possible, of course, that none of the samples were in fact sensitive to GA_3. Despite the lack of significance, however, the fact that, in the majority of cases, the GA_3-treated samples did have higher levels of activity, argues against this. The presence of alpha-amylase in endosperm slices incubated without GA_3 could have two explanations. Alpha-amylase was present in whole grains and it would therefore be expected that some of the endosperm slices would contain the enzyme. In addition, it is possible that alpha-amylase could have been produced during incubation. In the absence of measurements from unincubated slices it is not possible to answer this question. Avalon, Brock and Apollo all developed sensitivity to GA_3. In 1989, Apollo was later than the other cultivars in acquiring germinability and it is interesting to note that the onset of GA_3-sensitivity was rather later in this cultivar. Also of interest is the fact that GA_3-sensitivity began earlier in Fenman than in the other cultivars.

2.4.4 Water content, drying rate, and alpha-amylase activity

Caley (1986), found that, for glasshouse grown Fenman, water content began to fall just before maximum dry weight, and the present data supports this. Riffkin (1987), working with field-grown wheat, cv Sicco (in a location close to the current trial), shows water content decreasing from 37 DAA, and maximum dry weight attained at about the same time. Meredith and Jenkins (1975), working in New Zealand, found that there was a period of aproximately 26 days of relatively stable water content prior to the phase of rapid water loss. In contrast, in the present work, water content began to fall soon after maximum water content was reached. In Riffkin's (1987) data, there is little indication of a prolonged period of stable water content. This illustrates that individual physiological processes, as well as overall grain development, may vary in duration in different environments, and prompts doubts that the rate of moisture loss is as uniform for cultivars and seasons as Meredith and Jenkins (1975) suggest.

Water content, and rate of loss, particularly over the later stage of grain drying, have been implicated as factors in the control of PMAA in grains (Gale *et al.*, 1983, Gale *et al.*, 1987). The present experiment was only partially successful in its aim to induce different drying rates. The differences observed in 1989 may have been plot rather than treatment effects. Differences between treatments were greatest for Avalon, and the treatments should probably have been repeated in 1990. However, it was decided to repeat the treatments for Fenman only, because that was the only cultivar in which the treatments were associated with differences in alpha-amylase activity.

During development, grain water potential is generally lower than the rest of the plant, favouring the flow of assimilates to the grain. Assimilate uptake is associated with an influx of water, at least part of which must then be lost. In order to maintain assimilate uptake, there may be re-circulation of water back into the caryopsis, and Cochrane (1983) suggests a possible mechanism. This would help to protect the grain from water stress by allowing continued grain growth during drought conditions. Grain water potential has been shown to be relatively stable and independent from that of the surrounding grain structures (Barlow *et al.*, 1980; Brooks *et al.*, 1982). Little is known about the mechanism of water loss from grains. Both transpiration-driven (Lee and Atkey, 1984) and metabolically active (Meredith and Jenkins, 1975) processes have been proposed. The pathway of water loss is thought to be via the crease vascular tissue, through the region of xylem discontinuity, into the

xylem of the rachilla, and finally, via the xylem of the glumes, lemma and palea, into the surrounding atmosphere (Cochrane, 1983). She also suggests that water may be lost through the pericarp, via stomata found in the brush region of the caryopsis. The direct cause of the fall in water content is thought to be a block in the pathway of water uptake. Sofield *et al.* (1977) suggest that this may occur at either of two zones; at the xylem discontinuity at the base of the grain, or in the chalazal region. Cochrane (1985) suggests that pectic substances may block, or at least reduce the flow of water in, the xylem elements. With further water uptake blocked, water may be lost from the grain and both absolute and percentage water content decrease. It is possible that differences between cultivars during the later phase of grain drying are related to differences in grain or seed coat structure. There is a need for studies correlating ultramorphological developments with changes in water content in different cultivars and different environments. It is difficult to determine whether differences are due to slower loss of water, or increased uptake after rainfall. King (1984) found differences in grain water uptake between varieties, but no correlation was found between water uptake and grain colour, protein content, or pericarp or testa thickness.

Fenman had the slowest drying rate between 40 and 23% moisture and greater alpha-amylase activity than the other cultivars. However, the observation that, in 1989, the covered treatment had a slightly greater and the wetted treatment a slightly lower, rate of water loss than the control, but that both had higher levels of alpha-amylase suggest that, in Fenman at least, drying rate and alpha-amylase production are unrelated. For Avalon, the rate of water loss showed greater differences between treatments but there was no difference in alpha-amylase levels. It has been found that fungicide treatments significantly increase grain moisture of Avalon over a three week period, and that this is associated with a significantly reduced HFN (Gooding *et al.*, 1987). This effect of fungicide on HFN appears to be widespread and a review of trials shows that, in the majority of cases, fungicide application reduced HFN (Stevens *et al.*, 1988) The explanation put forward for these results is that fungicides prolong the duration of green leaf area, and so delay natural senescence and grain drying, which in turn affects alpha-amylase and HFN. An alternative explanation, suggested by the observation that prochloraz has sometimes had a more severe effect than other compounds, is that fungicides may have growth regulatory functions (Stevens *et al.*, 1988). If the effect of fungicides in reducing HFN is related to their effects on grain drying, it might

be expected that the differences in grain moisture found in Avalon in the present study would be associated with differences in alpha-amylase activity. That such an association does not occur is shown by the equally low levels of alpha -amylase in all treatments.

The lack of relationship between drying rate and alpha-amylase activity in the present work might be explained by the fact that all the drying rates were relatively fast. The suggestion that grain drying rate between 40% and 20% moisture may influence alpha-amylase production was based on an experiment using drying rates of aproximately 1.5 percent and 0.6 percent moisture loss per day for the fast and slow drying treatments respectively (Gale *et al.*, 1983). The "fast" drying rate was said to be similar to that of an average British growing season. It is difficult to determine drying rates from the data in Gooding *et al.* (1987); although the fungicide-treated samples usually have a higher moisture percentage, the rate of drying over the entire 25 day period shown was not actually very different between treated and untreated, at approximately 1 %/day. It is therefore more likely that their observed differences in HFN were associated with previous differences in grain moisture rather than with drying rate. The drying rates (40-23% moisture) of Avalon (1989) in the present study were close to or higher than Gale *et al.*'s (1983) "Fast" drying treatment, suggesting that drying was too fast to have an effect on alpha-amylase activity. If drying rate and alpha-amylase are related, it might be expected that a range of drying rates would produce a range of different alpha-amylase levels. However, it may be that there there is a "safe" drying rate, above which there is no damaging effect on alpha-amylase. Gale *et al.* (1983), in examining only two drying rates provide no information in this respect. Support is provided by the work of Kettlewell and Astbury (1990), however, who found no correlation between drying rate and pre-harvest falling number; their drying rates were all relatively fast compared to those of Gale *et al.* (1983).

It is difficult to determine accurately the exact time when the drying treatments were imposed in the work of Gale *et al.* (1983) and it is possible that the rise in alpha-amylase had been initiated before the treatments began. The fact that there was some increase in alpha-amylase activity, even in the fast drying treatment, suggests that the treatments did not actually initiate alpha-amylase production, but rather affected its subsequent level of activity. If this is so, it prompts another possible explanation for the lack of correlation between drying rate and alpha-amylase activity in the present study. As Fig 2.11 shows, there was never any indication of a rise in alpha-amylase after the steep decline

in "green" alpha-amylase, and therefore it is not surprising that different drying rates, and/or differences in moisture, had no effect.

Finally, of course, it may be that Avalon, Apollo, Mission and Brock are not susceptible to PMAA, whatever the environmental conditions. Genetic differences in susceptibility do exist (Flintham and Gale, 1988) but there is little information for these particular varieties. The study by Gooding *et al.* (1987), discussed above, related differences in HFN to differences in drying rate of Avalon. Although there is a strong relationship between alpha-amylase activity and HFN, there are other factors that may affect HFN, and in the absence of measurements of alpha-amylase it is not possible to attribute the reduced HFN to PMAA. If Avalon and Mission are resistant to PMAA, it further weakens the suggestion that PMAA was the principal cause of low HFNs in 1985. In that year, Avalon and Mission were the two most common varieties in the Home-Grown Cereals Authority survey at 37% and 10% respectively (H-G.C.A., 1985).

2.4.5 PMAA in Fenman

Neither of the explanations discussed above, ie; that alpha amylase activity will not differ between treatments with different, but (relatively) fast drying rates, or that drying rate will only have an effect if alpha-amylase production has already been initiated, can explain the results for Fenman, in which there was an initial increase in alpha-amylase in all treatments, relatively fast drying rates in all treatments, and differences in alpha-amylase activity in both the wet and the covered treatments in 1989. Whether these effects were in fact due to the treatments is debatable. Treatments were not replicated in 1989, and no effects were found in 1990, suggesting that the 1989 effects could have been due to plot rather than treatment. However, no plot effects were found in 1990, lending some support to the view that the effects were induced by the treatments. The apparent "effects" could also have been due to chance or normal variation. It is clear from all the present data that large variation does exist. However, such variation would be more likely to show up as random fluctuation, as in the 1990 data, than as a seemingly consistent trend, as shown by the lower activity of the control in 1989. There is no other obvious reason, apart from the imposed treatments, why the alpha-amylase levels in a particular section of a plot should be consistently different from those in an adjacent section. Plots had been initially checked for uniformity, using plant height as the variable, and no gradients, or abnormal patches, were observed. For these

reasons, the following discussion is based on the assumption that the differences in alpha-amylase observed in 1989, were due to the treatments. What is not in doubt however, is that Fenman is liable to produce prematurity alpha-amylase even during such "good" years as 1989 and 1990. In fact , H.-G. C. A. surveys show that, nationally, HFN's in 1989 and 1990 were the highest since records began.

Cornford and Black (1985) reported that Fenman was the only one of nine cultivars in which alpha-amylase levels increased towards harvest. In their work, a sharp rise in the level of the enzyme occurred at 44 DAA when grain moisture was approximately 41 %. In the present study, in 1989 the increase in alpha-amylase activity occurred between 45 and 49 DAA. During that period, moisture percentage fell from 34% to 30%, and from 38% to 32% for the wet and dry treatments respectively. In 1990, the rise in activity occurred between 48 and 56 DAA for all treatments and moisture percentage fell from about 40% to about 37%. Vertucci (1989) suggests that enzyme activity is diminished at low moisture levels. However, data from Caley (1986) indicate that enzymes may differ in their response to reduced water content. She found that while starch synthase activity was not affected by a reduction from approximately64 to 33 percent moisture, ADPG pyrophosphorylase activity was reduced to an extremely low level. The sensitivity of alpha-amylase synthesis to reduced water content is unknown, but Gale *et al.* (1983) found that extractable enzyme activity remained constant once about 20 % moisture had been reached. However, Cornford and Black (1985) harvested their trial at 19 % moisture, at which point there was no indication that levels of alpha-amylase had stabilised, although infrequent sample points make it difficult to determine accurately. In 1990, in the present study, daily samples, while showing fluctuation, tend to indicate that a peak level of alpha-amylase was reached at about 58 DAA, when moisture percentage had increased to about 39% again. The reason for this is unknown; according to Gale *et al.* (1983) it would be expected that levels of alpha-amylase would continue to rise until grain had dried to about 20% moisture.

It was not known whether the fluctuations in alpha-amylase levels observed in 1989 were real or were due to normal variability and it was hoped that the daily sampling in 1990 would provide an explanation. Fluctuation in levels of the early "green" amylase has been reported by Olered and Johnsson (1968) and Meredith and Jenkins (1973) but there are no reports of such daily changes in the level of alpha-amylase in mature grains. It is possible that re-

wetting of grains due to rainfall could renew alpha-amylase synthesis. However, given the variation found between individual grains in 1989, and the fact that there was apparently daily fluctuation in levels of alpha-amylase in 1990, the most likely conclusion must be that normal variation is the cause of the apparent rise and fall in activity.

Cornford and Black (1985) found that, while a sample of grains from 38 out of 40 ears had measurable alpha-amylase activity, the bulk of the activity was confined to grains from just two ears, and, within those ears, there was no obvious pattern of alpha-amylase activity. Such variability in individual grains has also been recorded in Maris Huntsman (Evers and Ferguson, 1979) and Mardler (McVittie and Draper, 1982). In sprouted grains, an assymetric distribution of alpha-amylase in noted, with a small proportion of the sample having very high activity (Mc Vittie and Draper,1982). It is suggested that this is a result of the rapid increase in alpha-amylase during germination. While the 1990 data indicate a similar rapid increase during prematurity alpha-amylase production found in the present work, data for individual grains in 1989 were only taken from 48 DAA, by which time significant levels of activity were already present.

Results for Fenman, in 1989, indicated that differences in grain drying rate did not explain the observed differences in alpha-amylase activity between the wet and covered treatments, and the control. An alternative explanation was sought. The wet and covered treaments both initially involved covering the plots with a polythene tent and it was thought possible that the change in microclimate (the polythene tent would have tended to increase temperature and humidity in the covered plots) might have been responsible for the observed effect on alpha-amylase activity. How this effect might be mediated is open to speculation; one possibility might be via changes in sensitivity to growth regulators. The individual grain data suggests that the wet and covered treatments increased the proportion of affected grains, and it might be that such grains differ in GA-sensitivity. Thus, the study of GA-sensitivity in 1990 was an attempt to investigate whether a covering treatment affected either the timing of the onset of GA-sensitivity, or the magnitude of the response. It was expected that the covered treatment would respond differently to the control and the wetted treatment, and that this would be reflected in higher levels of alpha-amylase activity in harvest-ripe grain. However, the results show that the onset of GA-sensitivity occurred at about the same time in all treatments, and there was no difference in alpha-amylase activity of whole grains. This does not,

however, rule out the possibility that the 1989 results may have been due to environmental effects on GA-sensitivity. There was variability in the response to GA_3, indicating that GA-sensitivity is not uniform throughout the population. The steep rise in alpha-amylase activity at 49-50 DAA in 1990 indicates that the timing of particular biochemical and/or physiological processes may be extremely critical. Thus, although "DAA" were used in an attempt to impose the 1990 covering treatment at the same stage of grain development as the previous year it may be that the critical stage was missed. Grain moisture at the time of treatment was higher in 1990 than 1989 (below).

Duration of covering treatment

	Date	DAA	"DAA"	Moisture %
1989	18/7-28/7	29-36	35-40+	59-49.7
1990	25/7-3/8	36-45	36-42	55.2-44.8

In conclusion, the two years of trials showed that Fenman was particularly susceptible to PMAA. The increase in alpha-amylase activity occurred over a rather narrow time period, and was unaffected by drying rate, although environmental effects earlier in grain development may have had an effect.

3. The effect of the *Rht* genes on PMAA and sucrose relationships.

3.1 Introduction.

3.1.1 Effect of the *Rht* genes on GA-sensitivity and alpha-amylase activity.

Numerous studies have shown that, in isolated aleurone layers, and in de-embryonated grains, GAs stimulate the production of alpha-amylase, and it is probable that GA_1 plays a similar role *in vivo* during normal grain germination. The dwarf wheat genotypes, Norin 10 and Tom Thumb, were found to be insensitive to applied GA with respect to stem elongation (Allan *et al.*, 1959). Initially it was thought that the two characters were controlled by different, but closely linked genes, and separate gene symbols were assigned for the dwarf (*Rht*) and GA-insensitive (*Gai*) characters. It is now known that a single gene, with pleiotropic effects, is responsible, and the *Gai* symbol has been dropped. Four different GA-insensitive dwarfing genes have been described (Gale and Youssefian, 1985). *Rht1* and *Rht2* are derived from Norin 10, and are located on chromosomes 4A and 4D respectively. They have very similar effects on plant height. *Rht3* is present in the genotypes Tom Thumb and Minister Dwarf, but its original source is unclear. It has been shown to be an alternative allele to *Rht1* on chromosome 4A (Gale and Marshall, 1976). It has a more severe dwarfing effect than *Rht1* or *Rht2*. Finally, an even stronger dwarfing gene, *Rht10*, has been identified in a Chinese variety, Ai-bian 1, on the short arm of chromosome 4D. Several other *Rht* genes have been described, but these are not associated with GA-insensitivity.

The GA-insensitive *Rht* genes described above exhibit a common shoot response. However, they differ in their effect on aleurone sensitivity. Incubated with GA_3, Norin-10-derived genotypes showed the normal response to GA for both whole grains, and de-embryonated half grains, whereas *Rht3*-carrying genotypes showed very little response (Gale and Marshall, 1973). The reason for this difference is unknown.

The *Rht3* gene has been associated with lower residual alpha-amylase levels in mature grain (Gale, 1976). However, Bhatt *et al.* (1976), found no notable differences in alpha-amylase levels of harvested grain of parental or segregating populations of crosses between *Rht3* and *rht* lines. Their trial was carried out in Australia, and it is possible that environmental "triggers" are required before genotypic differences become evident. In another, UK, trial *Rht3* reduced by 84% the number of grains exhibiting PMAA in near-isogenic lines of Maris Huntsman (Gale *et al.*, 1987). The relative amount of alpha-

amylase activity in affected grains was also reduced. The *Rht1* gene had a similar, but less pronounced effect, reducing affected grains by about 40% compared to the tall, *rht* genotype.

The effects of the *Rht* genes in reducing alpha-amylase activity, in both sprouted and unsprouted grains, is of obvious interest to plant breeders attempting to overcome the problems of sprouting damage. Flintham and Gale (1982) found that field-sprouted samples of *Rht3* lines contained 77% less alpha-amylase than *rht* lines, despite similar levels of sprouting, and they suggested that such a level of reduction would be enough to eliminate sprouting damage as a problem in the UK. Mares (1987b), however, calculated that alpha-amylase production by the scutellum (which he found to be unaffected by *Rht3*), during the initial stages of germination was sufficient to reduce HFN's below breadmaking standards and concluded that "*Rht3* would appear to be of little use in a sprouting tolerance breeding programme". To date, much of the plant breeding effort has been directed at conventional pre-harvest sprouting. However, the apparently widespread occurrence, and increasing importance, of PMAA means that genetic sources to overcome the problem will have to be sought. The disadvantages of *Rht3* are its extreme dwarfing effect, and its yield reduction, caused by reduced grain weight (Gale, 1976) and these will need to be overcome before it can be of commercial use.

3.1.2 Sucrose metabolism and the control of alpha-amylase activity.

It has been proposed that sucrose may be involved in the control of grain development (Duffus and Binnie, 1990). Embryo culture experiments indicate that the development and germination of embryos is influenced by their growth environment. For instance, high sucrose or amino acid concentration in growth media delayed *in vitro* premature germination of cultured barley embryos (Cameron-Mills and Duffus, 1980). This could be related to osmotic effects; ie if the surrounding medium is of lower osmotic potential than the embryo itself it will tend to withdraw water from the embryo. Dunwell, (1981) demonstrated that increasing the sucrose concentration of the culture medium resulted in a decrease in percentage water content of barley embryos. However, Barlow *et al.* (1983), using cultured wheat ears, found that while increased sucrose concentration in the culture media led to decreased water uptake, it also led to decreased transpiration, such that the measured water content in the ear was relatively unaffected.

Withdrawal of water may in itself prevent germination, by reducing water

below a threshold level required for germination. Alternatively, the effect may be mediated via ABA. It is well established that water stress increases levels of ABA in seeds, and that applied ABA can inhibit germination (King, 1982). It may be then that withdrawal of water stimulates ABA synthesis and thus prevents premature germination. Morris *et al.* (1988), examining the effect of low water potential on ABA content of cultured embryos, found that barley embryos increased their ABA content if cultured on a mannitol-containing medium, but not if they were cultured on a basal medium without mannitol. In contrast, ABA content of wheat embryos was not affected by either medium. It was suggested that both embryo ABA levels and the external osmotic potential are important in preventing germination, that the relative importance of each factor may differ between species, and that endogenous ABA levels in wheat embryos may therefore have been sufficient to prevent germination on the low osmotic potential mannitol medium (Morris *et al.*, 1988). Garçia-Maya *et al.*, 1990) also found no increase in ABA content in wheat embryos cultured on mannitol. Lee *et al.* (1989) cultured immature ears of wheat, and found that absence of sucrose from the medium considerably increased grain ABA content between 5 and 22 days after anthesis. However, this treatment also severely reduced grain weight, and, while the increase in ABA was not just due to a concentration effect (smaller grains) it is clear that these were not "normal" grains and the significance of the result therefore is doubtful. Xu *et al.*, (1990) concluded that, while both ABA and media of low osmotic potential (0.35M sucrose) prevented germination of alfalfa embryos, the fact that normal synthesis of developmental proteins only occurred on osmoticum indicates that osmotic potential may be the most important *in vivo*.

The above discussion relates to the control of germination of immature embryos. It is less clear whether sucrose plays any role in preventing pre-harvest sprouting or premature alpha-amylase production during the later stages of grain development. There would appear to be at least four possible ways in which sucrose and sugar metabolism may be involved. Firstly, the requirement for embryo hydration prior to germination would still remain, so presumably osmotic effects could still be important. Secondly, Duffus, (1990) has suggested that while sucrose may act as a protector of embryo membranes, it may crystallise out during slow drying, leading to membrane damage. Such damage would presumably affect membrane transport characteristics, and, indirectly, the physiological and biochemical processes involved in alpha-amylase production. Thirdly, it is possible that sugars may form an intracellular

glass during desiccation, thereby preventing diffusion and biochemical processes (Koster, 1991). Finally, it could be that sucrose is involved in the control of GA synthesis. Radley (1969) suggested that a block in sugar metabolism, by preventing the synthesis of GA, may be part of the mechanism maintaining low enzyme levels in ripe grains in humid conditions. There is little information on developing grains but studies of germination in mature grains provide some support for Radley's hypothesis. Smith and Briggs (1980) suggest that, in normal germination, gibberellin release from the embryo begins when the sugar content of the embryo has been reduced by metabolism initiated by imbibition. Alpha-amylase, synthesised as a result of GA-stimulation, is considered to be the primary enzyme of starch breakdown. Sucrose and other sugars produced by the breakdown process are transported to the growing embryonic axis, and, if they accumulate, are thought to suppress GA supply. Radley (1969) showed that GA-like activity was reduced in isolated scutella incubated on agar containing 0.5% and 2% glucose. Whole embryos were not affected, indicating that sugar utilisation by embryonic axes prevented inhibitory levels being reached. In addition, high levels of sucrose in the endosperm appear to depress the ability of the aleurone to respond to GA (Briggs and Clutterbuck, 1973). Jones and Armstrong (1971), showed that isolated aleurone layers from 4 day germinated grains could still respond to added GA, and suggested that the apparent lack of response in intact grains was due to an inhibition of alpha-amylase release caused by the osmotic effect of the build up of sugars. This conclusion has been questioned by Gepstein and Ilan (1974) who found that osmotic potential in germinating barley changed little with time, and that reducing the osmotic potential (by transfer to dry conditions) did not affect alpha-amylase production. Briggs and Clutterbuck (1973) suggest that the apparent reduced response could be due to osmotic effects and/or turnover of alpha-amylase, with destruction equalling or exceeding synthesis. If such a sugar-controlled "feed-back loop" (Smith and Briggs, 1980) exists in germinating grains it is possible that a similar system may control the premature production of alpha-amylase in developing grains.

The objectives of this study therefore were twofold: firstly, to examine the effect of the *Rht* genes on PMAA, with particular reference to GA-sensitivity during grain development, and secondly, to examine the relationship between sucrose and alpha-amylase activity.

3.2 Materials and Methods.

3.2.1 Plant Material.

Five near-isogenic lines (*rht, Rht1, Rht2, Rht1+2, Rht3*) of Maris Huntsman were drilled on 10/10/89 in loam soil in Horse Park field, Bush Cereals Trials Centre. Plots (11x2m) were unreplicated. Previous cropping, seed rate, fertiliser, and pesticide applications were as outlined in Section 2.2.5. Plots were sampled from anthesis until harvest. Measurements were made of fresh and dry weight (2.2.2), extractable alpha-amylase activity (2.2.4), GA-sensitivity (2.2.8), and sucrose content.

3.2.2 Sucrose Extraction and Determination.

Endosperms (10) and embryos (20) were dissected out on to moist filter paper in petri dishes on ice. Each set of tissues was homogenised on ice, in 1 ml ice-cold 0.05 M perchloric acid using an Ultra Turrax T25 electrical homogeniser (Janke & Kunkel, Germany). Ice-cold distilled water (3ml) was added and the mixture further homogenised. The mixture was centrifuged (Sigma 202 MK) for 10 min at 5000 *g* and 0-4°C. The supernatant was decanted, the pellet re-extracted in 0.5ml ice-cold perchloric acid (0.05 M), and 1.5 ml ice-cold distilled water added. The mixture was centrifuged as before. The two supernatants were combined and the pH adjusted to 6.8 using 5M KOH and 0.05 M KH_2PO_3. The solution was weighed, the total volume calculated, and then stored at -20°C. Sucrose was determined using test combination kits (Boehringer Mannheim GmbH, Sandhofer StraBe 116, 6800 Mannheim 31, Germany). The method is based on the following reactions:

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{hexokinase}} \text{Glucose-6-phosphate}$$

$$\text{Glucose-6-phosphate} + \text{NADP} \xrightarrow{\text{G-6-P-DH}} \text{gluconate-6-phosphate} + \text{NADPH} + \text{H}^+$$

$$\text{Sucrose} + \text{H}_2\text{O} \xrightarrow{\beta\text{-fructosidase}} \text{Glucose} + \text{Fructose}$$

The sucrose extract (50μl) was mixed with 100μl β-fructosidase solution in citrate phosphate buffer (pH 4.6), and incubated for 5min at 37°C, before adding 500μl triethanolamine buffer (pH 7.6) containing NADP and ATP, and 850μl distilled water. The absorbance at 340nm was read after 3min (Beckman DU6 spectrophotometer). Hexokinase and glucose-6-phosphate dehydrogenase suspension (10μl) was then added and mixed and the absorbance at 340nm read

after 15 min. A second sample was similarly treated but without the initial addition of β-fructosidase. NADPH, measured by its absorbance at 340 nm, is stoichiometric with the amount of glucose present, and sucrose concentration was calculated from the difference in glucose concentrations with and without enzymatic inversion (β-fructosidase step). Results are the mean of three separate extractions, and are expressed as the concentration (mM) of sucrose per tissue, using the mean water content.

3.3 Results.

3.3.1 Grain Growth and Development.

A very similar pattern was observed for all five lines. Date of anthesis was 23rd June 1990 for all lines, and grain development continued at a similar rate throughout the trial (Fig 3.1). "DAA" lagged behind actual DAA initially, but warmer, drier weather from 13th July (Fig 2.1) increased the rate of morphological-development and by 33 DAA "DAA" were in agreement with, or more advanced than, actual DAA. From 40 DAA, considerable variation in the rate of grain development was observed within plots, masking possible differences between lines. Although *Rht 1* was slightly more advanced at 44 DAA, *Rht3* reached 51 "DAA" three days earlier than the other lines at 51 DAA.

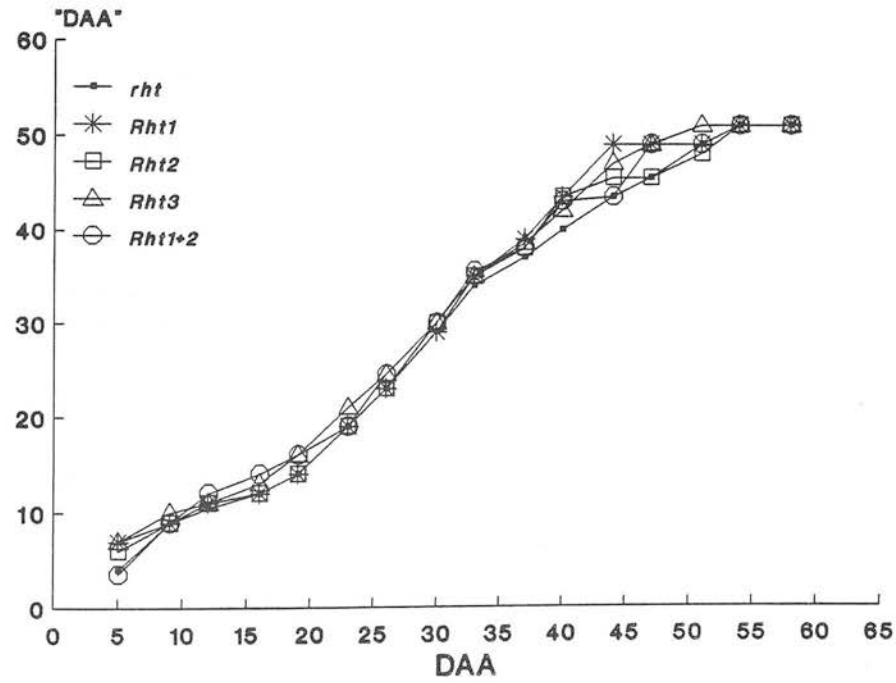


Fig. 3.1 Relationship between developmental age ("DAA") and chronological age (DAA) for lines of Maris Huntsman.

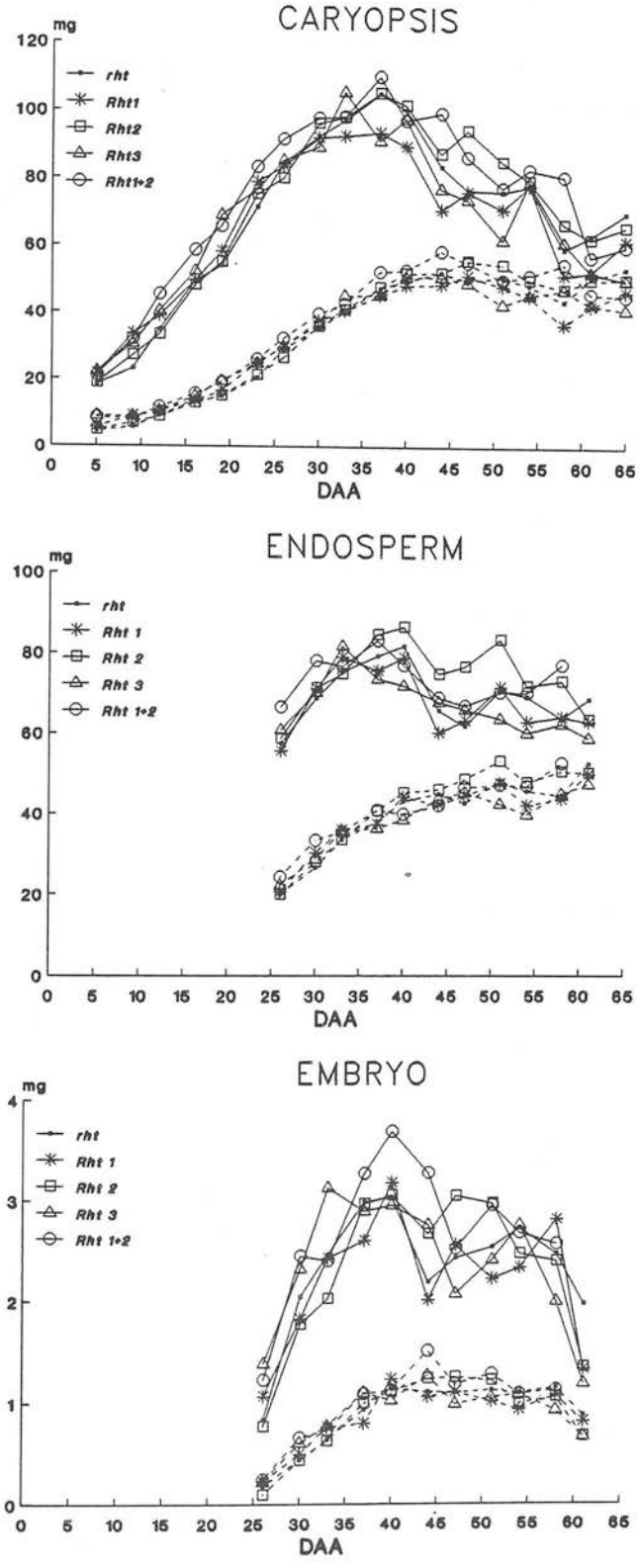


Fig. 3.2 Changes in fresh (---) and dry weight (- - -) of caryopses, endosperms and embryos during grain growth and development in 1990.

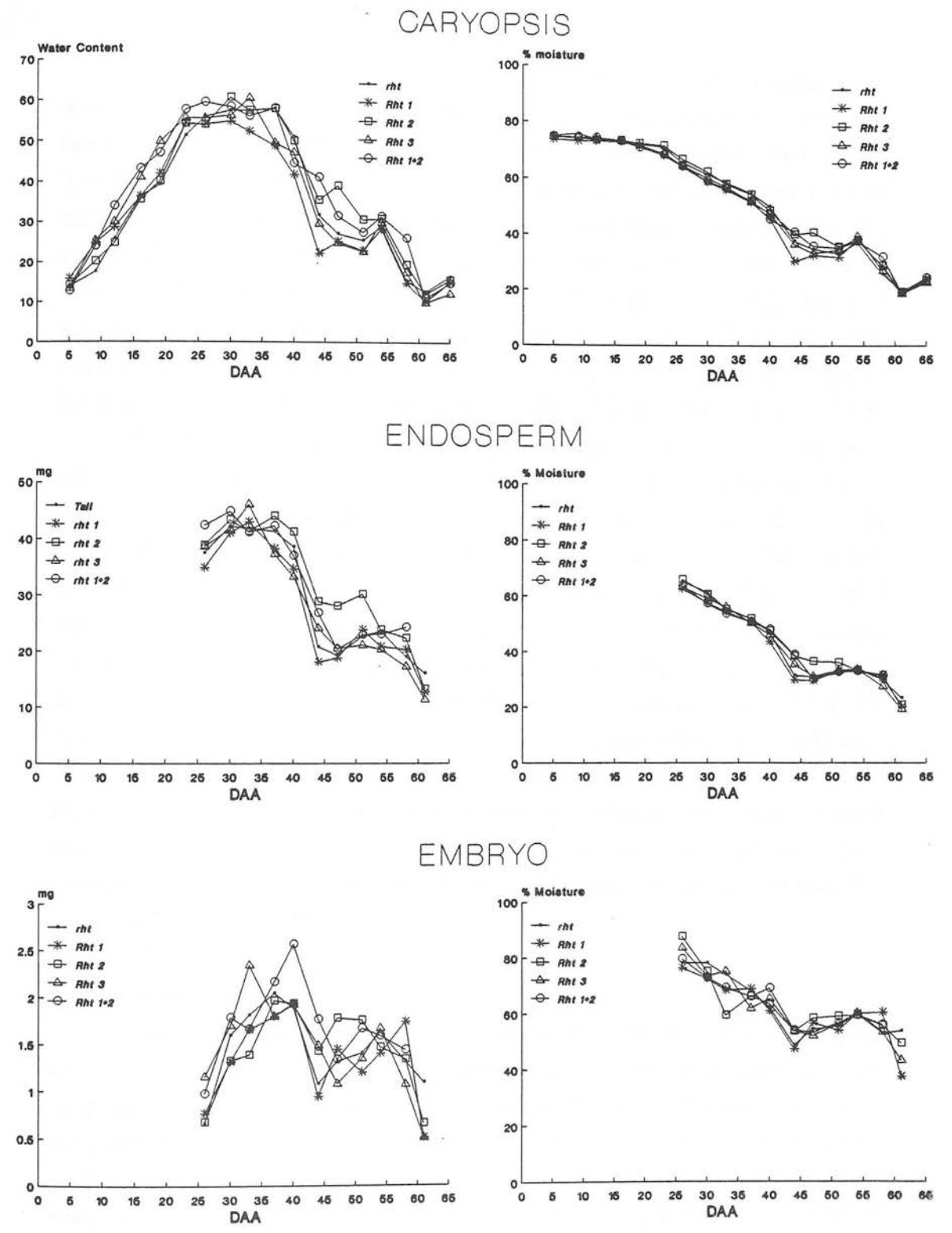


Fig. 3.3 Changes in water content (left) and percentage moisture (right) of caryopses, endosperms and embryos during grain growth and development in 1990.

No consistent differences in caryopsis fresh and dry weight were observed between lines (Fig. 3.2). Maximum fresh weight occurred at about 37 DAA (37-40 "DAA") and maximum dry weight at about 40-44 DAA (44-47 "DAA"). Changes in endosperm fresh and dry weight were similar to those of caryopses (Fig. 3.2). *Rht 2* had a greater endosperm fresh weight between 41 and 51 DAA (although 95% confidence limits overlapped with *Rht1+2* at 44 DAA). Embryos increased in fresh weight from 25-40 DAA (Fig. 3.2); *Rht 1+2* was heavier between 37 and 44 DAA but data was quite variable and 95% confidence limits overlapped. Dry weight of embryos increased until about 44 DAA and then remained fairly steady until a slight fall between 58 and 61 DAA.

Changes in water content and percentage moisture for caryopses, endosperms and embryos are shown in Fig. 3.3. Caryopsis water content was at a maximum between 30-33 DAA and decreased rapidly between 37 and 44 DAA. Water content then increased (*Rht1* and *Rht2*), or decreased less rapidly (*rht*, *Rht3*, *Rht1+2*) until 51 DAA when all lines gained water. A final period of rapid water loss from all lines occurred between 54-61 DAA. Endosperms showed a similar pattern of rapid loss, gain, and final steep loss of water. *Rht2* had a higher water content than the other lines between 37 and 51 DAA (although 95% confidence limits of *Rht 1+2* overlapped between 37-44 DAA). There were no consistent differences in embryo water content between the lines. It can be seen that all embryos maintained a higher percentage moisture than the corresponding caryopsis or endosperm. Embryo moisture percentage remained above 50% until the last date at which separation from the endosperm was possible.

3.3.2 Sensitivity to GA_3.

Sensitivity to GA_3, indicated by increased levels of extractable alpha-amylase, was observed in all lines from 48 DAA (Fig. 3.4), although one replicate of *rht* had a high level of alpha-amylase activity at 41 DAA. Generally, replicates responded similarly in showing higher levels of alpha-amylase activity in the presence of GA_3. The exceptions to this were *Rht3* at 55 DAA in which only one replicate responded, and *Rht1+2* at 59 DAA where two replicates responded. Although responses were similar qualitatively, the level of response varied, leading to a lack of significance in some cases. Significant differences ($P=0.05$) between plus and minus GA_3 were recorded for *rht* at 55 DAA, *Rht1* at 48 and 55 DAA, *Rht 3* at 59 DAA and *Rht1+2* at 52, 55 and 62 DAA. The greatest response to GA_3 was observed in *Rht1*, the least in *Rht2* and *Rht3*.

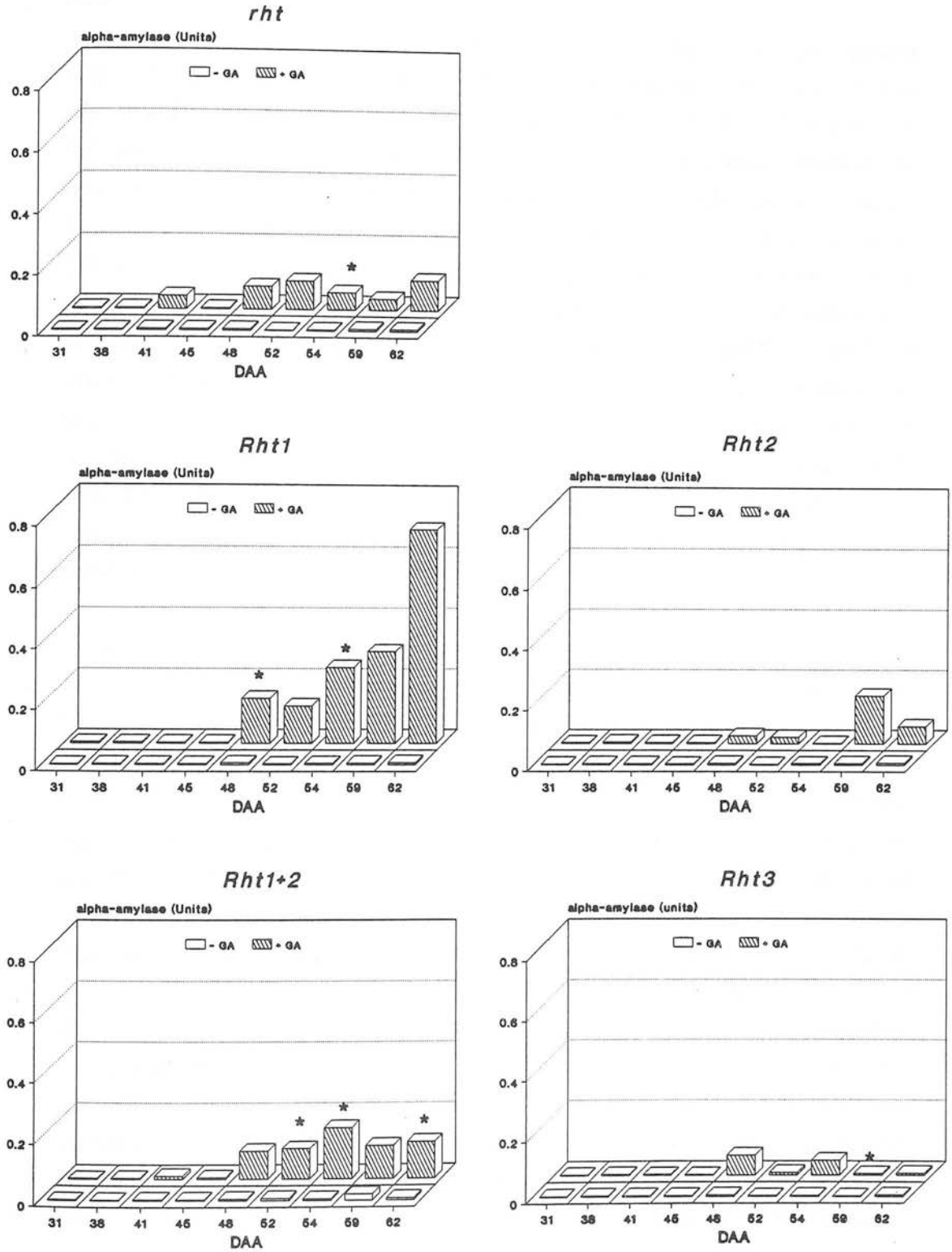


Fig.3.4 Alpha-amylase activity in endosperm slices incubated with or without GA_3. (* indicates significance at $p = 0.05$)

3.3.3 Alpha-amylase activity.

Alpha-amylase activity was high in caryopses during early development (Fig.3.5) but fell steeply between 30 and 37 DAA. Activity continued to fall in *Rht3* and *Rht1+2* and remained low until harvest (65DAA). *Rht1* had similarly low levels of activity apart from a rise at 54 DAA. Alpha-amylase levels in *rht* rose from 37 DAA, and activity remained consistently higher, though fluctuating, until harvest. In *Rht2*, activity rose and fell, rising again at 51 and 61 DAA. Lower levels of alpha-amylase activity were detected in embryos (note m units). Activity in *rht* embryos rose steeply between 44 and 47 DAA and remained at a higher level than the other lines until 54 DAA. Levels of extractable alpha-amylase were extremely low in the embryo compared to the rest of the grain, comprising, for example, from 1.2% (*Rht3*) to 8.6% (*Rht1*) of the total caryopsis alpha-amylase at 58 DAA. Calculated on a dry weight basis (Fig. 3.6), however, alpha-amylase activity of embryos and caryopses is within a similar range, apart from *rht* between 47 and 54 DAA when embryo levels are relatively higher.

3.3.4 Sucrose concentration.

Sucrose concentration was higher in the embryo than the endosperm throughout the duration of the experiment (Fig.3.7). For embryos, there was a general trend of an increase in sucrose concentration until around 44-47 DAA, followed by a decrease in all five lines. Sucrose concentration subsequently rose again in *Rht1*, *Rht2* and *Rht3*, but levelled off in *rht* and *Rht1+2*. Endosperm sucrose concentration varied to a lesser extent, though a small increase occurred in all lines, except *Rht1+2*, at 44-47 DAA. One of the aims of the study was to examine possible relationships between sucrose concentration and alpha-amylase activity. The preceding data is therefore re-presented in Fig. 3.8 to show alpha-amylase levels in the caryopsis and corresponding sucrose concentrations in the embryo and endosperm. In general, embryo sucrose concentrations were beginning to increase at the time when caryopsis alpha-amylase activity was decreasing from the early peak of "green" pericarp enzyme, but there was no obvious relationship between subsequent changes in alpha-amylase activity and sucrose concentration in either the embryo or endosperm.

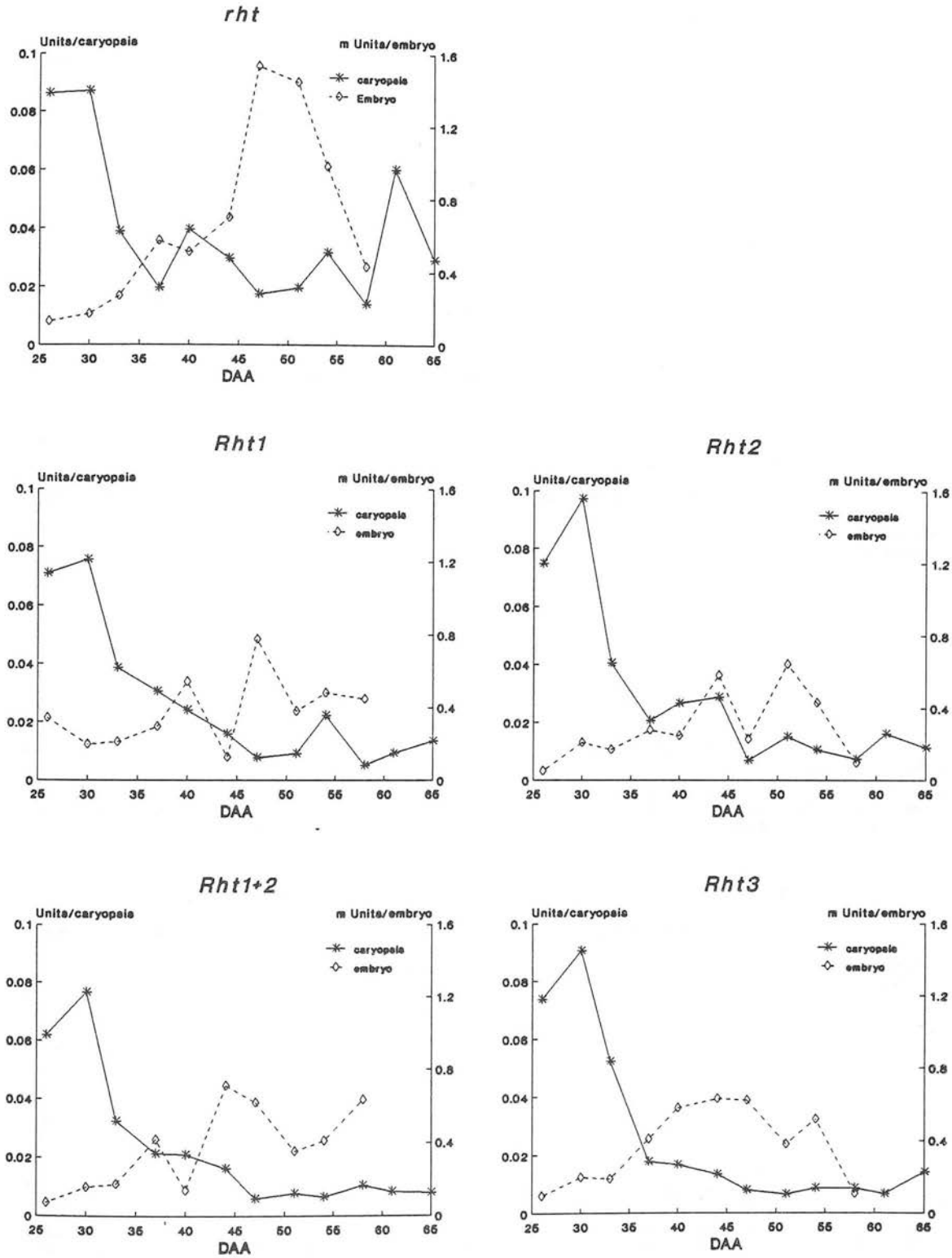


Fig. 3.5 Alpha-amylase activity of caryopses (*) and embryos (◊).

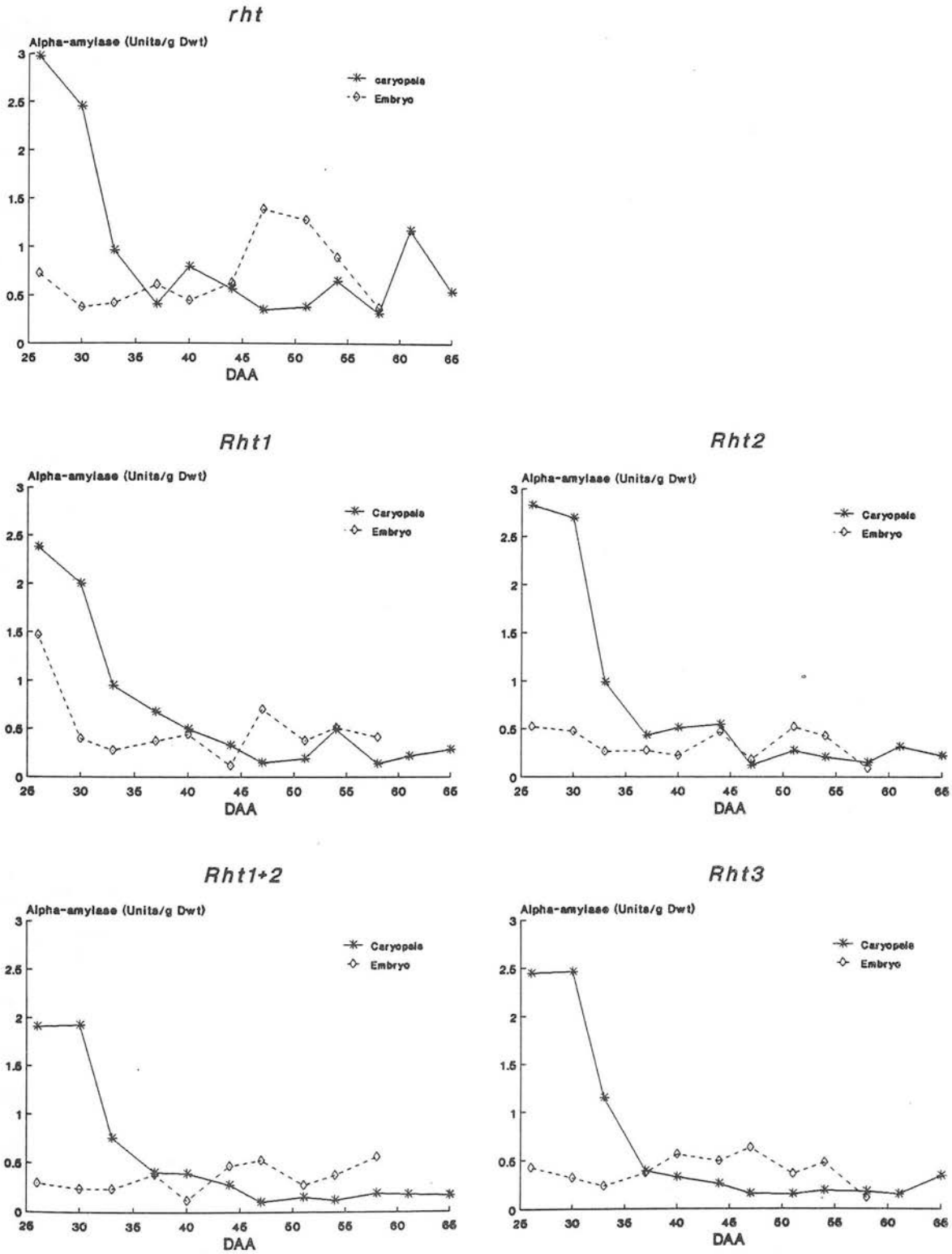


Fig. 3.6 Alpha-amylase activity (Units/g Dwt) of caryopses (*) and embryos (◊).

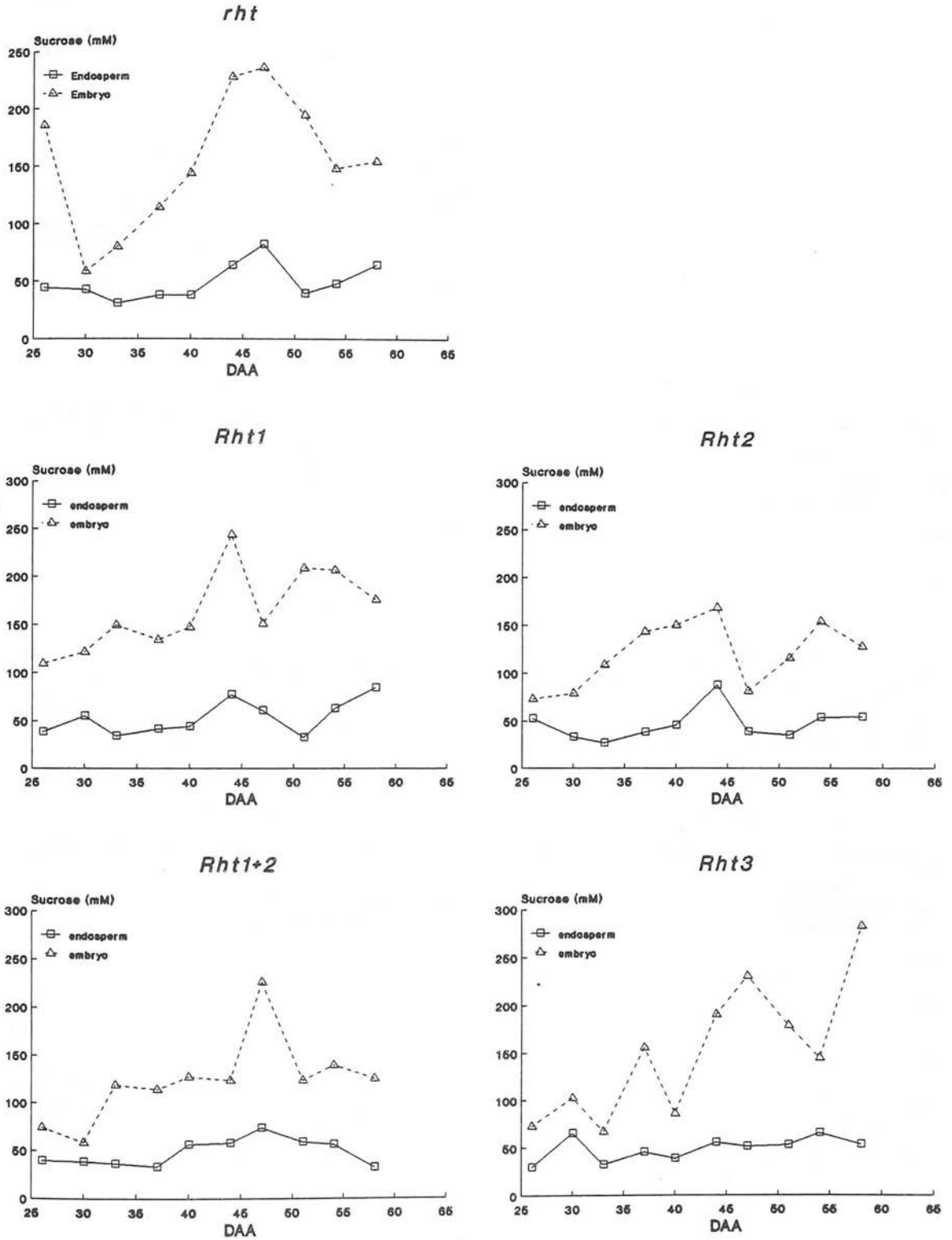


Fig. 3.7 Sucrose concentration of endosperms (□) and embryos (△).

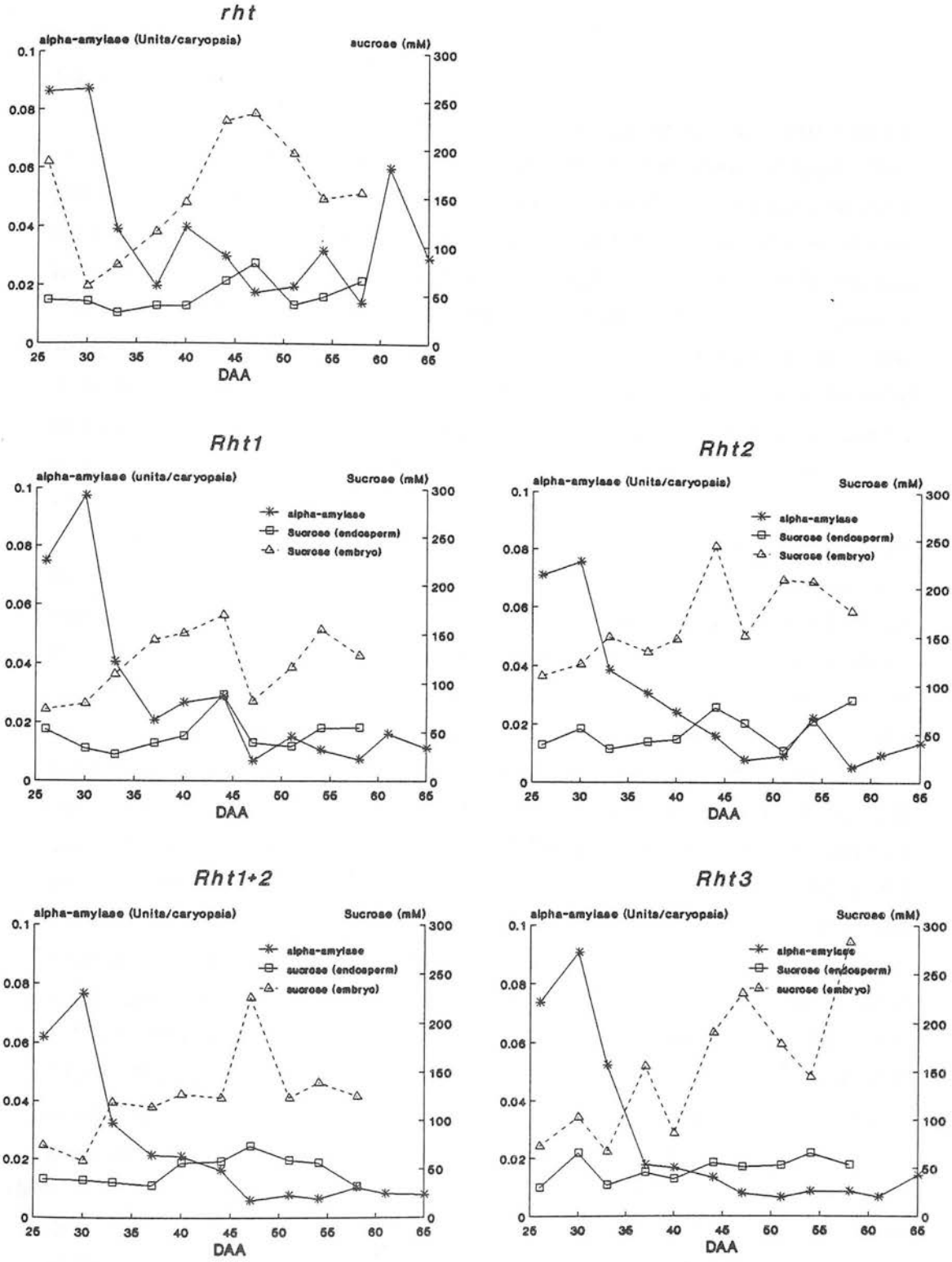


Fig. 3.8 Relationship between sucrose concentration of endosperms (□) and embryos (△), and caryopsis alpha-amylase activity (*).

3.4 Discussion.

3.4.1 Effects of *Rht* genes.

Previous studies have indicated that the *Rht* genes have a negative effect on grain weight in comparison to the tall, *rht* genotype (Pinthus and Gale, 1990; Borrell *et al.*, 1991). Flintham and Gale, (1983) showed that this was due to an increase in grain number in the dwarfs, with consequent increased competition for assimilates. They suggested that the increased grain set in distal florets was due to reduced dominance of the higher-yielding basal florets. In the present study, final grain dry weights are not greatly different, although *rht* is the heaviest and *Rht3* the lightest. Statistical comparisons are not strictly valid because these were single plots, and it is possible that plot effects may be masking any genotypic effects. Grain numbers were not recorded in the present study.

In 1977, a severe sprouting year, *Rht3* reduced grain alpha-amylase levels by 77%, while in 1980, a better year, alpha-amylase in unsprouted grain was reduced by approximately 83% (Flintham and Gale, 1982). This effect on PMAA was shown to be due to a reduction in both the number of affected grains, and in the relative amounts of alpha-amylase in affected grains (Gale *et al.*, 1987). Individual grains were not assayed in the present work, and the proportion of affected grains is therefore unknown. However, in comparison to *rht*, alpha-amylase activity at harvest (65DAA) was reduced by 54%, 61%, 51% and 73% by *Rht1*, *Rht2*, *Rht3* and *Rht1+2* respectively. *Rht3* did not have as great an effect as was expected. This is possibly due to the exceptionally good weather conditions prevailing during grain development in 1990. Previous reports of the effect of *Rht3* on PMAA were from field trials in 1982 and 1985 (Flintham and Gale, 1982; Gale *et al.*, 1987), when the mean UK HFNs were 162 and 252 respectively, compared to 334 for 1991 (H-G.C.A., 1982, 1985, 1990). If this is so, it indicates that *rht*, *Rht1* and *Rht3* differ in environmental sensitivity, with *rht* producing increased levels of alpha-amylase even in a good year, while *Rht1* only does so in a bad year such as 1985, and *Rht3* does not show increased levels. This environmental sensitivity may or may not be related to their differing GA-sensitivity. GA-sensitivity has been induced in the aleurone of *Rht3* by a 5°C preincubation treatment and it was suggested that this was due to an increase in the number of effective hormone receptor sites (Singh and Paleg, 1984). ABA-sensitivity of wheat embryos has been shown to vary in plants grown at different temperatures (Walker-Simmons and Sesing, 1990). Thus it is possible that environment could modify the inherent GA-insensitivity

of the *Rht* genotypes or alter the balance between GA and ABA sensitivity.

The pattern of alpha-amylase development in Maris Huntsman is rather different from that of Fenman (Fig 2.11). There was no clearly identifiable stage at which alpha-amylase activity rose steeply; rather there is a fluctuating, but consistently higher, level of activity in *rht* compared to the other lines, from 40 DAA. In fact, in comparison to Fenman, levels of alpha-amylase in *rht* are very low (less than 2%) and it is only relative to the other lines that *rht* has "high" alpha-amylase. The higher value at 61 DAA in the *rht* line is an indication that Maris Huntsman may be similar to Fenman with respect to the extreme variation associated with PMAA. However, there is another possible explanation. Later samples of Maris Huntsman, alone of the varieties studied in 1990, contained some visibly sprouted grains. The numbers of sprouted grains in the 10-ear samples were as follows:

	61DAA	65DAA
rht	0	0
Rht1	2	0
Rht2	2	0
Rht3	0	1
Rht1+2	2	1

Such grains were not used in the assays, but it is possible that others, in the earliest stage of germination, may have been overlooked, and contributed to higher enzyme levels.

Although some studies have found no alpha-amylase in immature wheat embryos (Garçia-Maya *et al.*, 1990), others have detected very low levels (Marchylo et al., 1980), and the present results support this. The alpha-amylase assay used in the present study is extremely sensitive and is able to detect activity as low as 0.01 mU/ml of extract (McCleary and Sheehan, 1987). However, the possibility of contamination of the embryo samples by other grain tissues cannot be ruled out. The fact that alpha-amylase activity in embryos and caryopses is similar on a weight for weight basis does not necessarily mean that small amounts of contaminating tissue would be too small to affect the result greatly. Alpha-amylase activity is unlikely to be evenly distributed throughout the caryopsis. In particular, the relatively high level of activity in the embryo of *Rht1* at 26 DAA could be due to contamination by pericarp tissue, the main source of alpha-amylase in the whole caryopsis at that stage. Similarly, the higher level of activity in *rht* embryos could be due to contamination from adhering endosperm tissue; Gale *et al.*, (1987) have noted that premature alpha-

amylase activity in Maris Huntsman is seen first, and accumulates more rapidly, at the embryo end of the grain.

If the higher level of activity in the *rht* embryos is truly embryo-derived then it suggests that the *Rht* genes also have an effect on scutellar alpha-amylase synthesis. It is not clear from previous studies whether GA-insensitivity, in *Rht3* genotypes, extends to the scutellum. Flintham and Gale (1982), observed lower levels of alpha-amylase activity in embryoless half grains of lines containing *Rht3*. Similar results were obtained during germination of whole grains, and they suggested that the GA-insensitivity of *Rht3* appeared to be common to both the aleurone and the scutellar tissue. In another study, however, lower levels of alpha-amylase activity in incubated, *Rht3*-carrying, embryos were attributed to insensitivity in aleurone tissue partially surrounding the scutellum (Gale and Marshall, 1975), with the implication that the scutellum itself was sensitive to GA. Mares (1987b), found that presence of the *Rht3* gene had no effect on alpha-amylase production by isolated germ tissue (embryo plus scutellum), nor on the initial rate of alpha-amylase production by whole grains. The different conclusions of these similar studies can possibly be explained by the fact that Mares (1987b) began sampling sooner than Flintham and Gale (1982), and also more frequently, therefore obtaining a more accurate picture of the earliest stages of alpha-amylase production, when the scutellum is believed to be the source of the enzyme (Okamoto *et al.*, 1980). Chandler and Mosleth (1990) have demonstrated GA-regulation of high pI alpha-amylase production in excised scutellar tissue, and a similar experiment, using GA-insensitive lines would help resolve the differences between Mares (1987b), Flintham and Gale (1982), and the present study.

There is no information about GA-sensitivity during grain development and ripening of *Rht*-containing lines, but a number of studies have examined such responses in mature, harvested grain. *Rht3* has been shown to confer aleurone insensitivity (Gale and Marshall, 1973; 1975; Ho *et al.*, 1981). The separate effects of *Rht1* and *Rht2* have not been studied, but Norin 10-Brevor 14, which contains both genes, was sensitive to applied GA_3 (Gale and Marshall, 1973). All of these earlier studies suffer from the fact that they were based on comparisons of varieties containing the *Rht* genes, and it is likely that other genes, apart from *Rht*, may have influenced the results. Thus, different varieties, although they were all derived from Norin 10, showed a range of GA-response (Gale and Marshall, 1975). The present study, based on near-isogenic lines of Maris Huntsman, provides a clearer picture of the effects of the

different *Rht* genes.

Rht1 and *rht* appear to be qualitatively fairly similar in their response to GA_3, which is in agreement with earlier studies (Gale and Marshall, 1973), albeit with varieties. *Rht3*, likewise, behaved as expected, with little or no response to GA_3. What is interesting, however, is the difference between *Rht1* and *Rht2*, and the relatively greater responsiveness of *Rht1* compared to *rht*. Such results have not previously been reported, although a report by Gale and Marshall (1975), of the results of crosses between Norin 10-Brevor 14 (*Rht1+Rht2*) and Tom Thumb (*Rht3*) is of relevance. They found that both the F_2 and backcross families contained individuals with greater responsiveness than the Norin 10-Brevor 14 parent. There were also a larger number of individuals with a very low response than would be expected if Tom Thumb-derived *Rht3* was the only strongly insensitive gene. The present results suggest that the more responsive lines may have carried *Rht1* and some of the less-responsive individuals *Rht2* rather than *Rht3*. The results for *Rht1+2* are intermediate between *Rht1* and *Rht2* which suggests that the two genes have an additive effect. This is borne out by their effect on plant height; individually they each produce a semi-dwarf phenotype, but together they reduced height to the same level as *Rht3*.

There is another possible explanation for the difference in GA-sensitivity between *Rht1* and *Rht2*. Black *et al.*, (1983) suggested that a critical water content must be reached before the aleurone cells acquire sensitivity to gibberellin. *Rht2* had a higher water content than the other lines between 37 and 51 DAA, and this might be related to its later acquisition of sensitivity. However, there was little indication from the other lines that there was a critical moisture level. Acquisition of GA-sensitivity occurred at moisture levels between 36% (*Rht1+2*) and 32.7% (*Rht1*). In the previous experiment (see 2.4.3), moisture level at the first indication of GA-sensitivity varied from 40.7% (Fenman, Control) to 36% (Brock). Of course, these are whole-grain values and the moisture level of the aleurone layer itself might be different.

It has been shown that *Rht1* reduces levels of alpha-amylase activity in mature, unsprouted grains (Gale *et al.*, 1987) and the present study confirmed this. The mechanism for this reduction is unexplained. The relatively greater aleurone responsiveness of *Rht1* compared to the other genes suggests that GA insensitivity is not involved. In the previous experiment (see 2.3.9) it was shown that while Avalon and Brock exhibited sensitivity to GA_3 this was not associated with premature alpha-amylase production. However, it might be that the

response, in both experiments, would have been different at a lower concentration of GA_3. Gale and Marshall (1973) showed that the response to GA_3 of whole grains of Norin 10-Brevor 14, and the *rht*-carrying Minister, depended on the concentration, with an optimum at about 10^{-4} M GA_3. A further experiment, using half grains and a wider range of GA_3 concentrations, showed that the minimum GA_3 concentration for a definite response varied from less than 10^{-9} M for Minister, to greater than 10^{-7} M for Norin 10-Brevor 14 (Gale and Marshall, 1975). While these results suggest that there is variation between the *Rht* genes in sensitivity to low concentrations of GA_3, dose-effect studies on isogenic lines are needed to confirm the result.

If the genes do differ in sensitivity to low levels of GA it is possible that variation in endogenous levels of GAs might be responsible for observed differences in alpha-amylase activity. In vegetative tissue, all *Rht* dwarfs are characterised by high endogenous levels of GA_1 (the normal GA of vegetative tissues), indicating a block to GA utilisation (Gale and Youssefian, 1985). However, Gale *et al.*, (1987) report little difference in the levels of GA_{54}, the normal seed development GA, in 18 DAA caryopses from isogenic lines carrying *rht*, *Rht1* and *Rht2*. It is not known, however, if the lines differ in the amount, or type, of GA in older grains. In cereals, there appears to be an early peak in unknown GAs just after anthesis, a larger peak of GA_{54} at about the time of maximum grain volume, and, occasionally, a smaller peak of unknown GAs towards maturity (Lenton and Gale, 1987). This last peak appears to be dependent on ripening conditions or maturity, which has led to suggestions that it may be involved in PMAA (Lenton and Gale, 1987). However, measurement of GA_1 and GA_{54} in developing grains of Maris Huntsman indicated no relationship with increasing alpha-amylase levels (Gale and Lenton, 1987).

Gale and Marshall, (1973) showed that incubated whole grains of both Norin 10-Brevor 14 and Minister responded in a similar way to GA_1, GA_3 and $GA_{4/7}$. Again, the independent effect of *Rht1* is unknown, but it appears as if variation in sensitivity to different GAs is not responsible for the reduced levels of alpha-amylase in *Rht1* compared to *rht*.

Although Flintham and Gale (1982) believed that it was "probable" that PMAA was GA-mediated, it is clear from the above discussion that the exact role, if any, of GA in the production of alpha-amylase in unsprouted grains is still largely unknown. The effects of the *Rht* genes in reducing alpha-amylase in mature grains, and the late peak in GAs suggest that it may be involved. On the other hand, the GA-sensitivity shown in half-grains of *Rht1* and *Rht2* as well as

rht suggests that other factors are also involved. It should not be thought that GA is an absolute requirement for alpha-amylase synthesis in grain tissues. Wheat embryos appear to be able to produce low pI-isozymes of alpha-amylase independently of added GA, though it is not known to what extent endogenous GA is important (Garçia-Maya et al., 1990). Marchylo *et al.* (1980) found that the composition of high and low pI isozymes in incubated embryo-scutellum tissues was similar in both the presence and absence of GA_3. Nicholls (1982) demonstrated alpha-amylase synthesis in endosperm halves in the absence of GA_3, although later reports suggested that endogenous GAs might be present (Nicholls, 1987). In addition, there is no evidence that the early, pericarp, alpha-amylase is controlled by GA (Radley, 1976a). In the study discussed above, Marchylo *et al.* (1980), found that 32 DAA endosperm-aleurone tissue produced low pI "green" alpha-amylase when incubated in the absence of GA_3. In conclusion, it seems that, to quote Gale and Lenton (1987), we "cannot assume that the sequence of events occurring during PMAA is the same as that during normal germination".

3.4.2 Sucrose relationships.

With the lack of any consistent correlations it is not possible to draw any firm conclusions about the relationship between sucrose concentration and alpha-amylase activity. However, a number of points can be made. The study confirmed Duffus and Binnie's (1990) finding that the embryo had a higher sucrose concentration than the endosperm, and also that the embryo moisture percentage was higher than that of the endosperm. It is possible that the relatively high embryo sucrose concentration may contribute to the difference in percentage moisture. Thus, if water is lost from the grain along a concentration gradient (Cochrane, 1983) the presence of sucrose in the embryo will tend to hold water in the embryo tissues. Alternatively, the embryo may have a different water-loss mechanism from that operating in the rest of the grain, and this might explain its higher water percentage. In either case, the outcome is the same, with the embryo being surrounded by tissues that are drier than itself. Morris *et al.*, (1991) have shown that the embryo maintains a consistently higher water potential than the caryopsis throughout grain development until just before harvest. They also note that the difference in water potential corresponds to the osmotic potential previously found to inhibit germination of cultured embryos. The water potential difference is probably most important during the earlier stages of grain development when water content is high

throughout the whole grain. Once dehydration begins, the amount of water available for movement from the endosperm to the embryo will be rapidly reduced, thereby lowering the risk of premature germination. In addition, dormancy will probably be the main factor suppressing germination by this stage.

It is still possible however, that sucrose may play a role in the onset of PMAA. Briggs and Clutterbuck (1973) reported that increasing sucrose concentration repressed alpha-amylase production in incubated, bisected barley grains, with virtually no enzyme formation at 100 mM sucrose. If GA_3 was then added, alpha-amylase was produced, but at a lower level than incubations with GA_3 but without sucrose. It was suggested that sucrose had a threefold effect: preventing embryo alpha-amylase production, suppressing endogenous GA supply, and, at the highest sucrose levels, depressing the ability of the endosperm to respond to GA. In the present study, the endosperm sucrose concentration in developing grains was about 50-60 mM. However, making an assumption that GA is involved in PMAA, if the "feed-back loop" suggested by Smith and Briggs (1980) and discussed above, does operate in developing grains, the embryo sucrose levels might be sufficient to prevent GA release. If this is so, then it would be expected that a rise in alpha-amylase activity would be preceded by a fall in embryo sucrose content. The present data provides little evidence to support this. In *rht*, embryo sucrose concentration does decrease from 47 DAA, followed by an increase in caryopsis alpha-amylase activity between 58 and 61 DAA. However, the initial rise in alpha-amylase activity, between 37 and 40 DAA, was at a time when embryo sucrose concentration was increasing. The lack of correlation, between sucrose concentration and alpha-amylase activity, in the other lines is not surprising. Even if embryo sucrose levels did influence GA synthesis, the GA-insensitivity of the lines would tend to mask any effect. This of course, prompts the observation that the GA-insensitive lines of Maris Huntsman were not the most appropriate plant material to use for the sucrose study, unless it was thought that sucrose was actually involved in the insensitivity mechanism. Ideally, isogenic lines varying in their sucrose concentration should have been examined for possible differences in alpha-amylase. For instance, high sugar mutants of maize had reduced germination compared to normal lines (Styer and Cantliffe, 1984). In the absence of such genotypes in wheat, comparisons between varieties with different sucrose concentrations could have been made. The disadvantages of such an approach have already been discussed; even if a

correlation between sucrose and alpha-amylase were found, the possible confounding effects of other genes would limit the findings.

Finally, sugar reserves are common in the embryos of many seeds, and are usually rapidly depleted during the first 24 h of imbibition (Bewley and Black, 1983), suggesting that they are the initial energy source for the expanding cells of the embryonic axis. It is possible that this is the major reason for the observed difference in sucrose concentration between the embryo and the endosperm.

In conclusion, the effect of the *Rht* genes in reducing PMAA was confirmed. Differences in GA-sensitivity during grain development were demonstrated between the isogenic lines. In particular, differences in response between *rht*, *Rht1* and *Rht2* suggest that the variation in GA-sensitivity cannot entirely explain the observed effects on premature alpha-amylase activity. Differences in sucrose concentration between the embryo and the endosperm were observed but appeared unrelated to alpha-amylase activity. It is concluded that the accumulation of sucrose in the embryo serves primarily as an energy store, but may also be involved in maintaining a water potential difference between the embryo and the endosperm.

4. Environmental effects during grain development and ripening on alpha-amylase activity in harvest-ripe grains.

4.1 Introduction.

Several studies have shown that environment modifies the level of pre-maturity alpha-amylase produced by susceptible cultivars. For example, a "slow" drying environment (15/10°C, 92% RH) was shown to produce about three times higher levels of alpha-amylase compared to a "fast" drying environment (15/10°C, 56% RH) in the variety Snabbe (Gale *et al.*, 1983). In another study, the Australian cultivar, Spica, was grown in New South Wales, Australia in 1987 (warm, dry) and 1988 (hot, dry) and in Cambridge, UK, in 1987 (cool, wet). Alpha-amylase activity increased in all samples from 40 DAA, with the highest levels recorded in samples grown in Cambridge, and the lowest in the 1988 NSW samples (Mares and Gale, 1990). In 1985, a year in which low HFNs in the UK have been attributed to PMAA (Flintham and Gale, 1988), the weather was cool and moist from 600-900 degree-days post anthesis, whereas in 1989, the period from 600-900 degree-days was characterised by above average temperatures and below average rainfall, and HFNs were high (Hough, 1990). Other studies, more specifically concerned with conventional pre-harvest sprouting, also reveal possible environmental effects on alpha-amylase activity. Thus, Nielsen *et al.*, (1984) report that high diurnal temperature differences, and low rainfall in the period from soft dough to one week after physiological maturity were both associated with low alpha-amylase activity in hard white winter wheat. Results reported earlier in this thesis (section 2.3.8), indicated that environmental effects during grain development, as opposed to grain drying, might affect alpha-amylase levels in Fenman. The present study, therefore, examines the effect of environmental treatments at different stages of grain development and ripening, on alpha-amylase activity in harvest-ripe grain of Fenman.

4.2 Material and methods

Winter wheat cv Fenman was drilled (as a spring crop) on 30/3/90 in loam soil in Crofts field, Bush Cereals Trials Centre. Seed rate was 220 kg/ha, and 65 kg/ha each of P_2O_5 and K_2O were applied in the seedbed, with top dressings of 60 kg/ha N applied on 4/4/90 and 30/4/90. Previous cropping was winter wheat, potatoes, spring barley and winter barley. Pesticides were applied as follows:

Fungicides	Calixin	11/5/90
	Tilt Turbo	13/6/90
Herbicides	Ally + Duplosan + Starane	25/5/90

On 4/6/90, when the plants were at about the five leaf stage, 400 plants were carefully dug up and potted into 7" pots. They remained in the field, within the surrounding plot area, until just before anthesis when all except 10 pots were moved into a well ventilated glasshouse. From there, pots were transferred to one of four different environments for ten day periods. The four environments were as follows:

1 Growth room, 15/9°C; 16h day, 8h night; 80-90% RH

2 Growth room, 21/18°C; 16h day, 8h night; low RH

3 In field, covered by "tent" (section 2.2.5)

4 In field, open, but within surrounding plot

A single pot was transferred to each environment on each day of the experiment. After ten days the pot was returned to the glasshouse. Ten pots remained in each environment, and in the glasshouse, throughout the experiment, and these are referred to as "control plants". The main stem of each plant was harvested at 69 DAA and frozen. Subsequently, twelve grains from the a and b florets of central spikelets were removed and used to estimate alpha-amylase activity (Section 2.2.4). An estimation of daily temperature was obtained by placing thermographs, previously calibrated, under the "tent" and in the glasshouse.

4.3 Results.

Extractable alpha-amylase activity of grains from the control plants is shown in Fig. 4.1a,b. Highest levels of activity were recorded in plants from the glasshouse, but plants from the cool and moist (15/9°C; high RH) environment had almost as high a level of activity. Apart from one plant, the warm and dry (21/18°C; low RH) environment was associated with relatively low levels of activity. Some of the control plants from the field environments were lost due to high winds towards the end of the experiment. The results for the remaining plants (Fig. 4.1b) show that, while variable, both open and covered treatments had a lower mean level of activity than the glasshouse or the cool and moist (15/9°C; high RH) environment. Results from the transferred pots were extremely variable (Fig. 4.1c-f) with no consistent trends relating alpha-amylase activity to either time of transfer or to a particular environment.

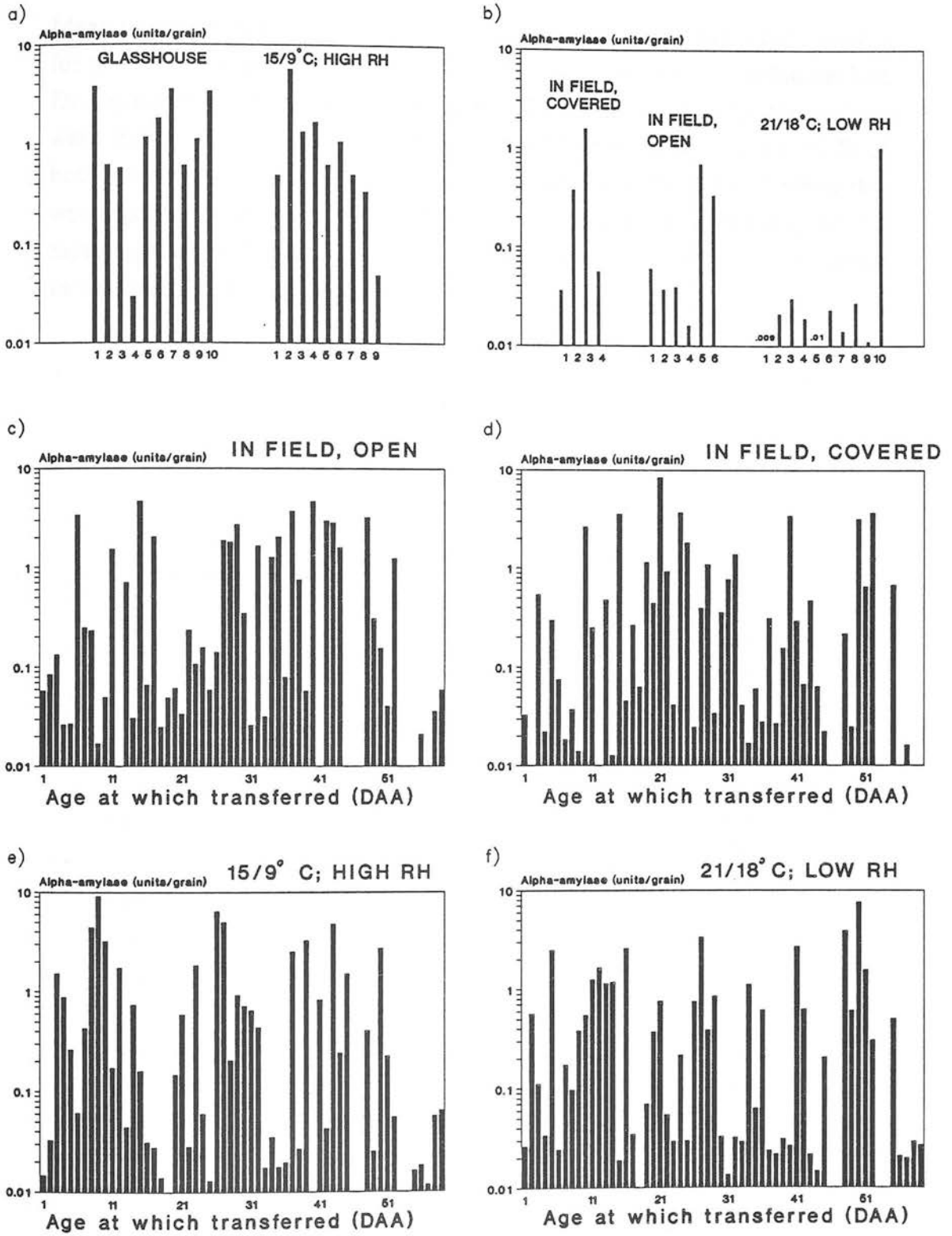


Fig. 4.1. Alpha-amylase activity in harvest-ripe grains from control plants (a,b), and plants subjected to ten day treatments (b-f).

Mean daily temperature observations are plotted in Fig. 4.2. Data were missing for some days, hence the plot of individual days rather than a continuous line. During the first half of the experiment, both the glasshouse and the covered plot were mostly warmer than the 21/18°C low RH environment. From 29 DAA both environments were generally cooler, apart from 39-42 DAA when they were again warmer. (Nb. This data should not be compared with Fig. 2.1; the thermographs were not enclosed and thus provide an estimation of direct, rather than shade temperature.)

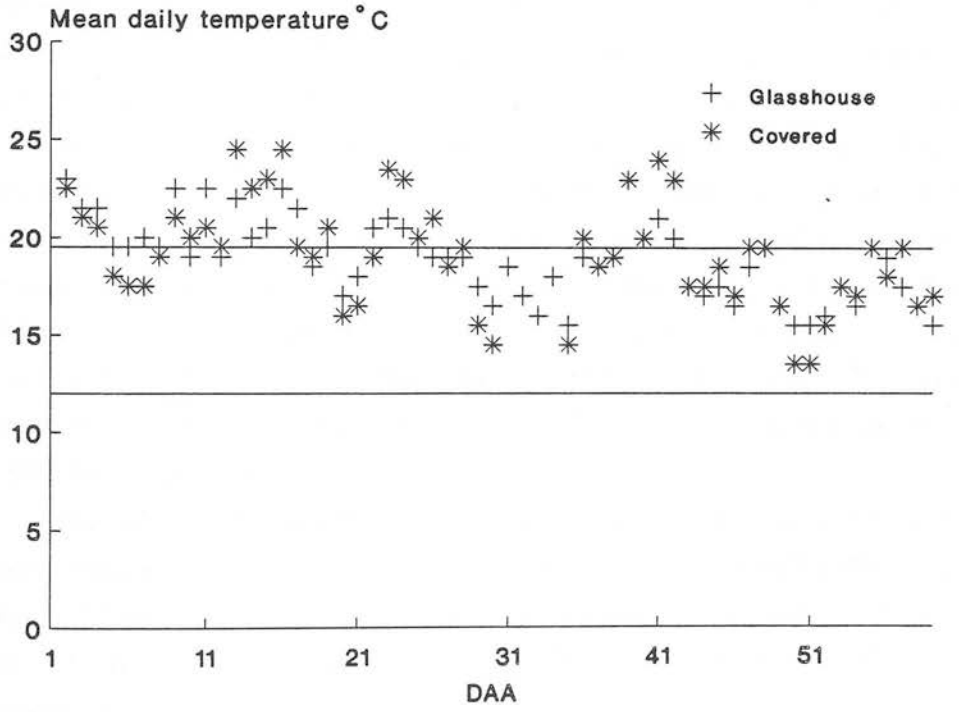


Fig. 4.2 Mean daily temperature in the glasshouse, and the covered environment. The upper solid line represents mean temperature in the warm, dry environment (21/18°C low RH), and the lower line the cool, moist (15/9°C; high RH) environment.

4.4 Discussion.

Comparing the control pots, it is clear that there are differences. The cool and moist treatment had higher levels of activity than the warm and dry treatment. This is in agreement with Gale *et al.*, (1983). However, the control plants from the glasshouse had even higher levels of activity. This last result makes it difficult to interpret the results of the ten-day treatments. It appears that the glasshouse environment somehow increases alpha-amylase activity, and thus it is impossible to know to what extent the measured alpha-amylase activity in transferred plants is due to the environmental effect of the glasshouse before and/or after the ten day treatment.

This apparent glasshouse effect is rather surprising and had not been anticipated when planning the experiment. Originally, the open field was to be the common environment, but it was thought that factors such as rainfall might modify any effect of the ten day treatments, and thus a well-ventilated glasshouse was chosen instead. Interestingly, Evers and Ferguson (1979) found that, in a survey of Maris Huntsman grown in different locations, glasshouse-grown ears had the highest level of activity. They suggested that this might be due to residual enzyme in the pericarp, but investigation of dissected grains showed that 80% of the activity was in the endosperm. It is possible that glasshouse conditions could hasten the onset of grain drying and thus allow the carryover of "green" amylase from the pericarp. However, if this were so, then it might be expected that the warm and dry environment would have a similar effect. In fact, control plants from that environment had generally very low levels of activity, and this suggests that the high activity of the glasshouse ears was not due to pericarp alpha-amylase.

How else might glasshouse conditions influence alpha-amylase activity? Watering was at soil level so there was no possibility of ear-wetting affecting the results. Artificial lighting was not used. It is difficult to identify a particular factor. Mean daily temperature was roughly similar to that in the covered environment.

There are reports of temperature effects on levels of the growth regulators ABA and GA. Radley (1976b) showed that higher temperatures were associated with increased GA in ears of wheat. In particular, she showed that it was ear temperature rather than plant temperature that was important. Pinthus and Meiri (1979) found that by reversing day and night temperatures (18/10°C --> 10/18°C) the growth habit of wheat was altered, with reduced stem and leaf elongation and greater tillering at 10/18°C. The effect was reversible

indicating reduced levels of endogenous GAs at cool day temperatures. A series of experiments by Nicholls (1982, 1983), investigating alpha-amylase production by de-embryonated barley, indicated that the requirement for GA_3 may be modified by the environment in which the grains were produced. Under certain conditions, so-called Type B grains were produced, which were able to synthesise alpha-amylase without the addition of GA_3 to the incubation media. Generally, higher growth temperatures tended to produce such grains. Summarising the studies, Nicholls (1987), suggested that there was more endogenous GA in the Type B grains, and that the environment might modify GA metabolism during barley grain development. Together, these three sets of results indicate that higher temperatures increase GA levels, and that it is the daytime temperature rather than the mean daily temperature that is important. If this is so, it has possible implications for the present study. Thus, daytime temperatures in the glasshouse may have been higher than in the other environments, leading to increased GA levels, and, assuming GA involvement in PMAA, increased alpha-amylase activity. However, the two controlled temperature environments provide conflicting results. For example, if daytime temperature does affect GA, then the warm, dry (21/18°C; low RH) environment would be expected to have higher GA levels than the cool, moist (15/9°C; high RH) environment. Yet, alpha-amylase activity was much lower in plants from the warmer environment. The assumption that GA is involved in PMAA is in fact unproven, and in the absence of firm evidence it is not possible to attribute differences in alpha-amylase activity to temperature-mediated differences in GA.

Environmental effects on other growth regulators, such as ABA, may also be important. Thus, Walker-Simmons and Sesing, (1990) have shown that ABA content of wheat embryos were higher at 25°C than at 15°C throughout most of grain development, but fell to similarly low levels at the final stage. The duration of grain development was considerably lengthened at the lower temperature with the result that ABA levels remained at a higher level for longer, and this was correlated with greater dormancy. Sensitivity to ABA was also greater in the embryos from grain produced at the cooler temperature. In the present study, no systematic observations were made on the duration of grain development, although it was clear that ripening was slower in the cool, moist (15/9°C; high RH) environment. This may have therefore resulted in greater dormancy in plants from this environment, and less in the glasshouse and other environments. However, there is no evidence to suggest that

dormancy is associated with reduced PMAA, or vice versa, that lack of dormancy results in increased susceptibility to PMAA. On the contrary, previous results (2.3.7) showed that Fenman, while susceptible to PMAA, had relatively high levels of dormancy. However, aside from its possible role in seed dormancy, ABA is also known to inhibit GA-induced alpha-amylase production (Fincher, 1989) and therefore it may still be important in the control of PMAA. This of course depends on whether GA itself is involved in PMAA. Until more is known about their involvement in the control of PMAA, the implications of environmental effects on levels of, or sensitivity to, both GA and ABA can only be speculative.

The effect of environment on PMAA seems to vary, even between susceptible cultivars. Thus, the Australian variety Spica produced alpha-amylase even in a hot, dry year, whereas Maris Huntsman, known to be susceptible to PMAA in UK conditions, had a very high HFN (indicating little or no alpha-amylase) in the same environment (Mares and Gale, 1990). Results reported in this thesis (section 2.3.8, 3.3.3) indicate that Maris Huntsman, with relatively low levels of alpha-amylase in the "good" year of 1990, differs in environmental sensitivity compared to Fenman, which produces high levels of alpha-amylase even in such a "good" year. National data on HFN support this observation, with HFN of Maris Huntsman varying from 142 in 1979 to 273 in 1984 (the lowest HFN for Maris Huntsman was recorded in 1977, but that was due to a high level of conventional pre-harvest sprouting) whereas that of Fenman was 135 in 1984 and 109 in 1986 (H.-G.C.A., 1977-1986). The present results from the warm, dry (21/18°C low RH) control group, in which alpha-amylase activity was generally low, indicate that in Fenman the "safe" threshold with respect to the environmental "trigger" inducing PMAA is higher than in other varieties.

It was suggested earlier (section 2.4.5) that environmental effects prior to grain drying might influence the proportion of affected grains, while later environmental effects, by prolonging grain drying, seem to affect the level of activity (Gale *et al.*, 1983). This might explain the lower levels of activity in the warm, dry (21/18°C low RH) control group compared to the glasshouse group, and the similar levels in the glasshouse and the cool, moist (15/9°C high RH) group. It might be that the warm, dry (21/18°C low RH) environment reduced both the proportion of affected grains, and, by rapid drying, the level of activity. The glasshouse environment somehow increased the proportion of affected

grains, but not the level of activity, while the cool, moist (15/9°C high RH) environment, by slowing the drying rate, increased the levels of activity in affected grains. If this were so, then the ears from the cool, moist (15/9°C high RH) environment would be expected to contain fewer affected grains, but with higher levels of activity compared to the glasshouse with the result that mean levels of activity per ear would be similar. If this hypothesis were correct, it might also be expected that plants transferred from the glasshouse to the cool, moist (15/9°C high RH) environment at the beginning of the grain drying period (say, 45 DAA) would have the highest levels of activity due to a high proportion of affected grains and a slow drying rate. There is no indication from the results that this is the case. Conversely, those transferred to the warm, dry (21/18°C low RH) environment at, say 30-40 DAA, would have a relatively lower level of activity due to a low proportion of affected grains and a fast drying rate in the glasshouse. Although the plants transferred at 34 and 36 DAA have higher levels, the rest of the plants from 29-40 DAA do have a generally lower level of activity. Since no assays of individual grains were carried out, this hypothesis could not be tested.

The levels of activity in grains from the present experiment were generally within the same range as field-grown Fenman from the same year (section 2.3), suggesting that neither spring sowing nor transplanting had any confounding effect on alpha-amylase activity. It was hoped that this experiment would enable the identification of any stages of grain development that were particularly sensitive to environmental effects. This information could then have been used to carry out detailed studies, over a narrower time period, resulting in a more efficient use of time. Although no such "critical" stages were identified, the study did show that cool, moist conditions were associated with higher levels of alpha-amylae activity than warm, dry conditions. It is possible that such effects are not solely due to variation in grain drying rate. It is likely that the production of alpha-amylase in mature, unsprouted wheat is under complex genotypic and environmental control and small changes in levels of, or sensitivity to, plant growth regulators may have a significant effect.

5. The use of ear culture to manipulate grain drying rate.

5.1 Introduction.

Evidence to date (see sections 1.7.4, 2.4.4) suggests that the rate of grain drying may have a significant effect on alpha-amylase activity in mature, unsprouted grains. Therefore, most of the experiments described in the present thesis were designed to provide information on the effects of various environmental variables, as well as genotype, on premature alpha-amylase activity. Three approaches were tried, with mixed results.

Covering and wetting treatments were largely unsuccessful in altering the grain drying rate in the field, but did, however, indicate that increased temperature and/or humidity during earlier stages of grain development might be associated with greater alpha-amylase activity in harvest ripe grains (2.3.8). In another experiment (4.3), field-grown plants were transplanted and transferred to different environments for 10-day periods during grain development and ripening. No consistent correlations of alpha-amylase activity with environmental treatment were found, possibly due to an unexpected "glasshouse effect" masking any treatment effects. In addition, effects of grain drying rate on alpha-amylase activity, using glasshouse grown plants transferred to controlled environment growth rooms, were investigated. Such studies (not reported here) were limited by the number of plants that could be accommodated in the growth rooms, by the lack of availability of the growth rooms for an extended period, and also by their reliability. In view of these difficulties, a method was sought whereby sufficient quantities of plant material, grown under the same conditions, could be dried in a reliable, controlled environment.

The technique of detached ear culture has been used to study developmental phenomena in caryopses under controlled conditions. Jenner (1968) originally cultured ears of wheat for relatively short periods (1-4 days) on sucrose solutions. However, for longer term culture, a full culture medium is required, and a suitable method was established by Donovan and Lee (1977, 1978). Ear culture has been used as a method of controlling the assimilate supply to the ear, in studies primarily concerned with grain growth, and, ultimately, crop yield (Donovan *et al.*, 1983; Jenner and Rathjen, 1978; Cervantes *et al.*, 1989a). Nicholls (1979, 1986b) used a similar method to study the effect of different metabolites on the development of GA-sensitivity in wheat grains. Studying the effect of water loss on the termination of grain growth, Caley (1986) utilised polyethylene glycol (PEG) in the culture medium

to manipulate grain water content. The objective of the present work was to develop a similar method in order to examine the effect of grain drying rate on alpha-amylase activity in near-isogenic lines of Maris Huntsman.

5.2 Materials and methods.

5.2.1 Plant material.

Field-grown ears of near-isogenic lines of Maris Huntsman were used. Details of husbandry etc. are described in section 3.2.1 Ears were sampled on 5/8/90 (43 DAA, 40 "DAA"). Each ear was cut below the flag leaf node, placed in a plastic bag, and removed to the laboratory. Stems were re-cut, and stood in sterile distilled water until required. The flag leaf was then removed and the exposed stems surface sterilized using a cotton wool ball soaked in 10% sodium hypochlorite. The stem was then cut, under sterile distilled water, just above the node. Each prepared ear was inserted loosely through a sterile cotton wool plug into a sterile 50 ml glass vial containing 25ml of sterile liquid culture medium with or without PEG. Cultured ears were maintained in racks in a shallow water bath (4°C; maintained by re-circulation of water from an external bath containing a cooling coil) within a Fisons 600H controlled environment plant growth cabinet. Temperature was 16/12°C, with an 18h photoperiod. RH was between 85 and 90%. Culture media were renewed every 3-4 days by transferring the ears to freshly sterilized vials and media. Initial preparation, and subsequent transfer, of ears was carried out in a laminar flow cabinet. Sixteen ears of each genotype (*rht, Rht1, Rht2, Rht1+2, Rht3*) were assigned to each of two culture media - Control medium and medium+PEG. Every 3-4 days, at the time of transfer, 3 ears were removed from each genotype-treatment combination. Four grains were removed from the central spikelets, weighed and dried to constant weight (2.2.2). The rest of the ear was frozen and, later, 5 further grains were removed from the central spikelets and assayed for alpha-amylase activity (2.2.4).

5.2.2 Ear Culture.

Stock solutions of culture media were made up as follows:-

Major elements A	9.8 g $CaCl_2.2H_2O$ per litre.
Major elements B	14.4 g $MgSo_4.7H_2O$ and 24 g KH_2PO_4 per litre.

Minor elements (made up by serial dilution)	6.2 mg H_3BO_3, 10.6 mg $ZnSO_4.7H_2O$, 0.25 mg $NaMoO_4.2H_20$, 0.025 mg $CaCl_2.6H_2O$, 22.3 mg $MnSO_4.4H_2O$, 0.83 mg KI, and 0.025 mg $CuSO_4.5H_2O$ per litre.
Iron solution	5.57 g $FeSO_4.7H_2O$ and 7.45 g Na_2EDTA per litre (Double distilled water).
Vitamin solution	4 mg Thiamine HCl and 1 g Myo-inositol per 100ml.

Culture medium (Control) was then made up as follows:- 50ml Major elements A, 50ml Major elements B, 10ml Minor elements, 5ml Iron solution, 10ml Vitamin solution, Glutamine (2.6089g) and Sucrose(40g), made up to one litre (pH 5). The solution was sterilized by membrane filtration (0.2 μm membrane). PEG-containing media was made up as above but with the addition of 50g/l PEG (avg. 8,000 MW) previously passed through an ion-exchange resin column (Amberlite MB-1). Batches of culture media were frozen in sterile containers prior to the start of the experiment, and defrosted overnight as necessary.

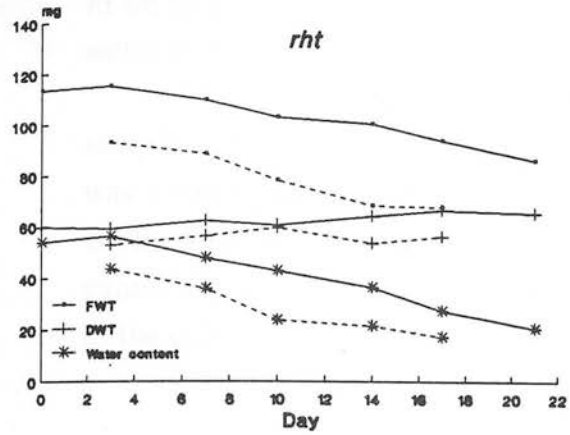
Statistical analysis

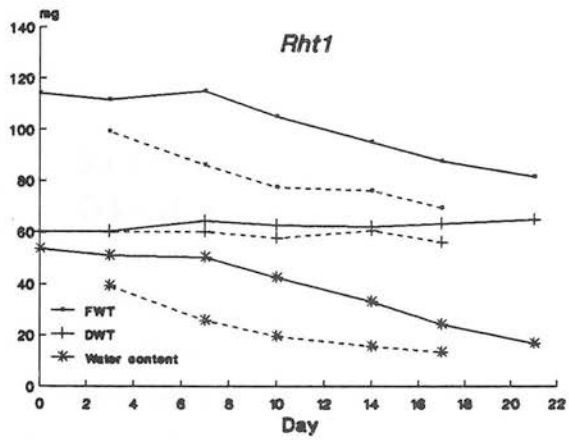
Data was analysed as a completely randomised design, with genotype, day (3-17), and culture media as factors. T-tests were performed where appropriate.

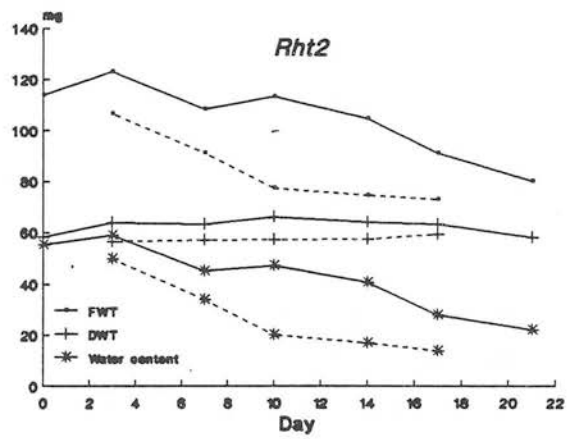
5.3 Results.

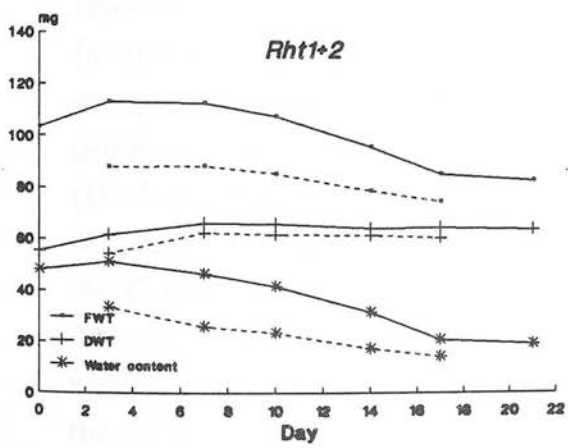
Sixteen ears of each line were cultured on either Control or PEG media, and 3 ears were sampled every 3-4 days. By day 17 of culture, ears on PEG appeared very dry and a final sample of 3 ears was taken. Ears cultured on the Control medium were not as dry, but microbial contamination of the media (Control only) was a problem by this stage. Of the 4 remaining ears, 2 were sampled and 2 were left for a further 4 days. Thus, while most data points represent the mean of 3 ears, those of the control at D17 and D21 are from two ears only.

Fig.5.1 shows changes in fresh weight, dry weight and water content over the duration of the experiment. Fresh weight initially showed an increase or remained more or less the same in caryopses from the Control treatment, while declining in those from ears cultured on PEG. From D7, fresh weight declined









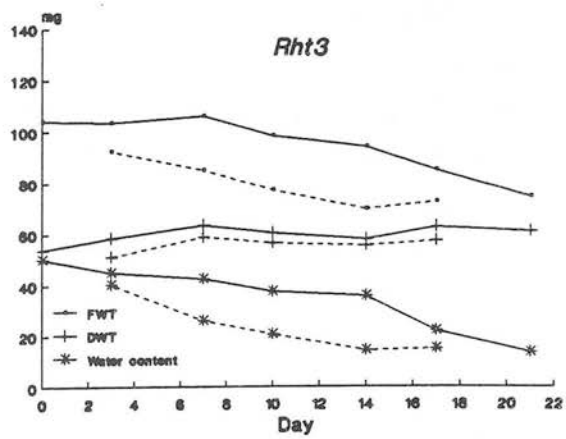


Fig.5.1 Changes in fresh weight, dry weight, and water content during ear culture on Control (—) or PEG (- -) media.

in all treatment combinations except for *Rht2*-Control in which an increase in water content was associated with a similar increase in fresh weight.

Caryopsis dry weight was higher in Control ears than PEG ears at all sample dates, in all lines, though the difference was never significant. There was a non-significant net increase in dry weight between D0 and D17 in all lines cultured on the Control medium. Caryopses of *Rht2*, *Rht1+2* and *Rht3* also gained dry weight on PEG, but *rht* and *Rht1* lost weight. Changes in dry weight of the cultured ears are compared with those from field samples (Table 5.1):

	Control	PEG	Field
rht	+6.73	-3.62	-1.17
Rht1	+2.97	-4.18	-6.01
Rht2	+5.44	+1.36	-1.71
Rht1+2	+9.13	+5.27	-12.35
Rht3	+9.27	+3.99	-8.04

5.1 Change in dry weight (mg/grain) between D0-17 (cultured ears) and 44-61 DAA (field trial)

Rht1+2 and *Rht3* cultured on Control media gained the most weight. These two lines, plus *Rht2*, also gained weight when cultured on PEG. In the field however, all lines lost weight, with *Rht1+2* and *Rht3* losing the greatest amount.

Caryopsis water content rose between D0-D3 in *rht*, *Rht2* and *Rht1+2* Control ears but subsequently decreased, apart from the increase in *Rht2* reported above. Culture media, day, and genotype all had a significant ($p=0.001$ or less) effect on water content. Water content of PEG-cultured samples was lower than the Control on all days, and for all genotypes. Within genotypes, the difference was significant ($p=0.05$) for *rht* on D10 and D14, *Rht1* (D7, D10), *Rht2* (D10, D14), *Rht3* (D14) and *Rht1+2* (D7). Water content of *Rht1* and *Rht3* Control, and all PEG-cultured lines decreased from D0, while the Control samples of *rht*, *Rht2* and *Rht1+2* showed a slight, non-significant increase, before decreasing in a similar way. At D17, caryopsis fresh weight and water content was still higher in the Control compared to the PEG medium, but the difference was not significant for any genotype. By D21, the final Control samples had a water content closer to, but still mostly higher than, the final (D17) PEG samples. An exception to this general observation was *Rht3* in which water content of caryopses from D21 Control ears was lower than those from D17 PEG ears. Overall, (days, and culture media), *Rht2* had a greater

water content than the other lines. Final fresh weight of caryopses from Control ears remained higher than those from PEG ears. This mostly reflects a higher dry weight in Control ears, though in *Rht2* it was due more to higher water content.

Percentage moisture is another way in which water content may be compared. There was an initial increase in moisture percentage in *rht*-Control, and a fall in all other genotype-treatment combinations (Fig.5.2). By D3, moisture percentage of *Rht1*-PEG and *Rht1+2*-PEG was lower than the other PEG-cultured lines and all the Control-cultured ears and on subsequent samplings, PEG-cultured lines always had a lower moisture percentage than their equivalent Control ears. At D7 the moisture percentage of PEG-cultured *rht* and *Rht2* was rather higher than the other PEG-cultured lines. From D10 onwards there was little difference in moisture between the lines cultured on PEG, but there were larger differences in the Control-cultured ears. *Rht3* lost very little moisture between D10 and D14, but then showed a steeper fall than the other lines between D14 and D17. *Rht1+2* showed a smaller decrease than the other lines between D17 and D21.

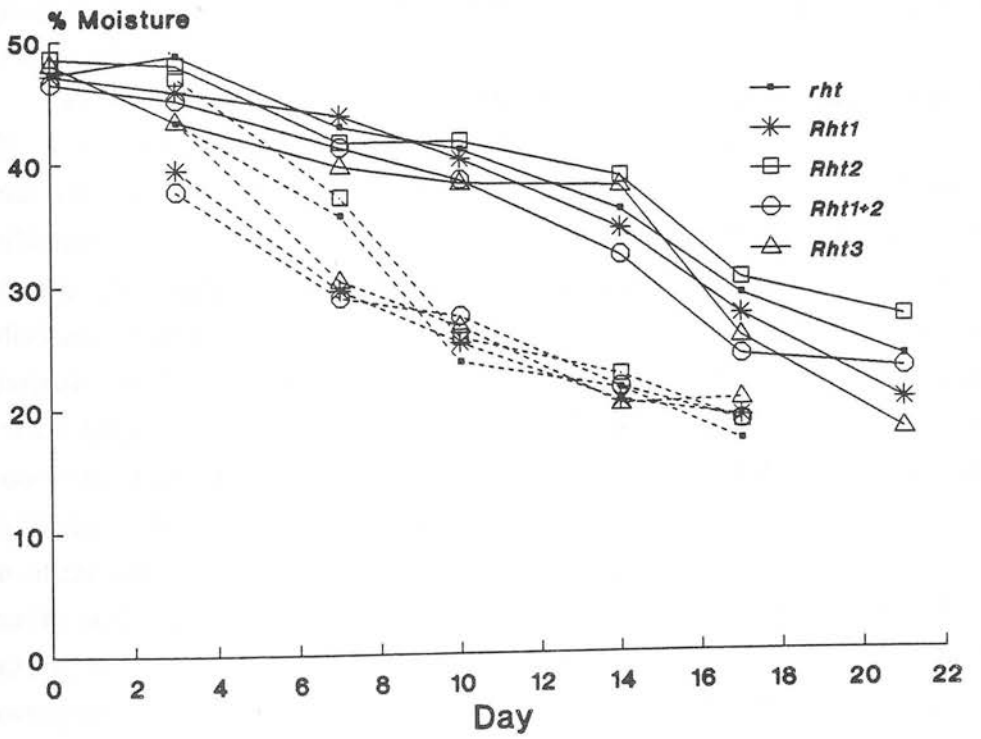


Fig.5.2 Changes in percentage moisture during ear culture on Control (—) or PEG (- -) media.

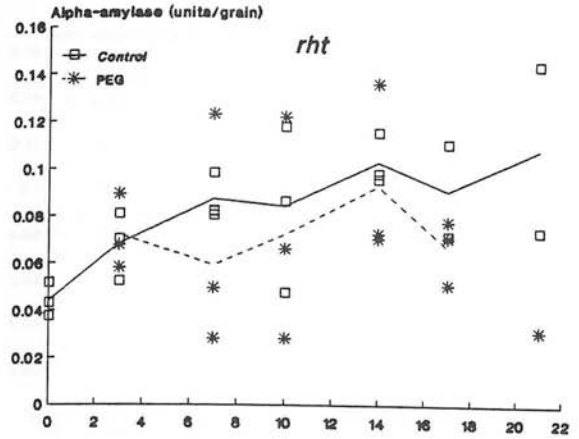
The rate of drying between D0 and D17 of culture was estimated using linear regression of moisture percentage on day (Table 5.2). The regression equations explained from 72% (*rht*-Control) to 91% (*Rht2*-PEG) of the variation in moisture.

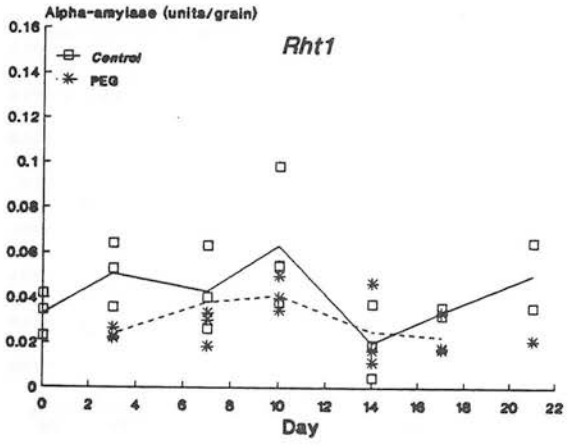
	Control	PEG
rht	1.04	1.87
Rht1	1.07	1.66
Rht2	0.927	1.93
Rht1+2	1.21	1.55
Rht3	0.989	1.73

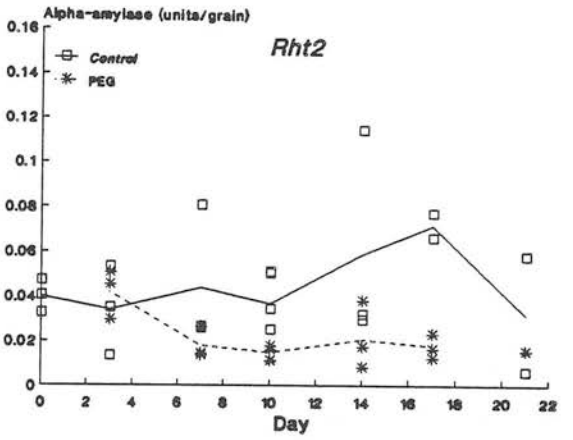
Table 5.2 Grain drying rate (percentage moisture loss/day) of ears cultured on either PEG or Control media.

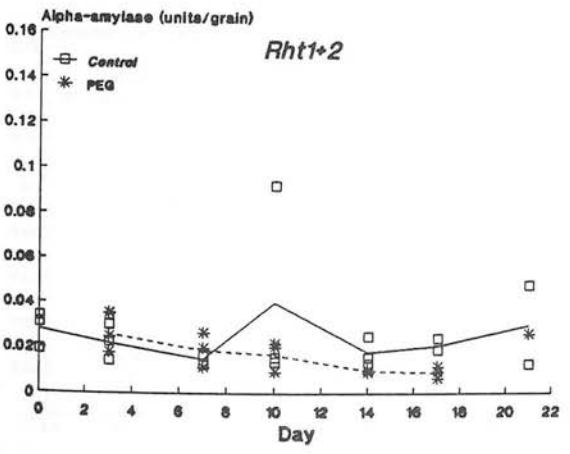
Grain drying rate was always higher in PEG-cultured ears. There appeared to be no relationship between genotype and drying rate; *Rht2* had the fastest drying rate among PEG-cultured samples, but was the slowest-drying of the Control samples.

Extractable alpha-amylase activity was plotted against sample day (Fig. 5.3). There was much variation between replicates, particularly in *rht*, *Rht1* and *Rht2*, and so the figures show both the mean (line) and the individual ear replicates (single data points) at each sample date. Although the mean lines indicate that alpha-amylase activity was generally higher in the Control, individual replicates show that some PEG samples, particularly in *rht*, also had relatively high activity. Overall, alpha-amylase activity was significantly ($p = < 0.001$) lower in caryopses from ears cultured on PEG, but for individual genotypes, and for any particular sample day, the difference was never significant. *Rht1+2* and *Rht3* had lower levels of alpha-amylase activity than the other lines and differences between Control and PEG samples were also smaller, although grains from one *Rht2*-Control ear had higher activity at D10. Plotting alpha-amylase activity against sample day (Fig 5.3) is possibly not the most appropriate way of examining the data. Apparent trends with time may be influenced by the difference in physiological age of the Control and PEG samples. Therefore alpha-amylase activity was plotted against moisture percentage for each individual ear in order to compare alpha-amylase activity of Control and PEG samples at a similar moisture level (Fig.5.4). The majority of









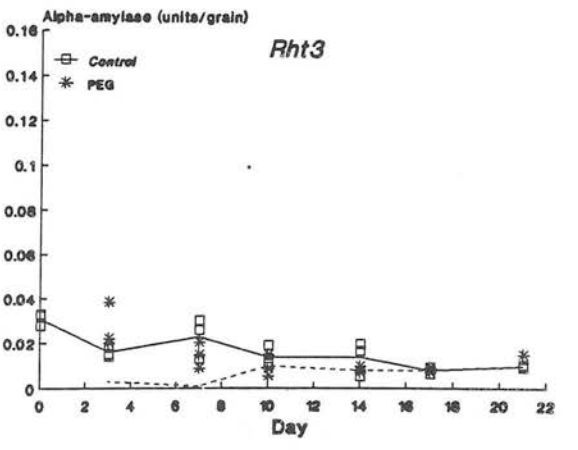
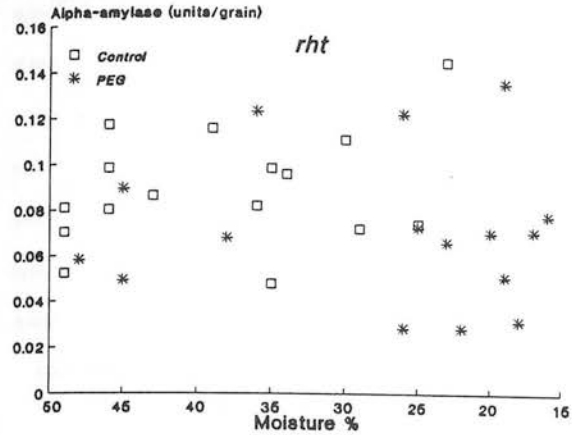
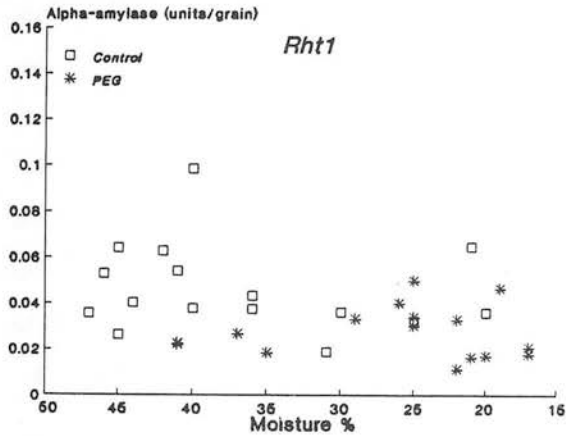
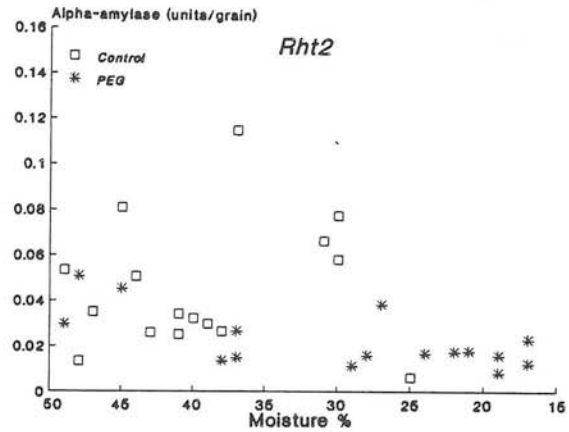
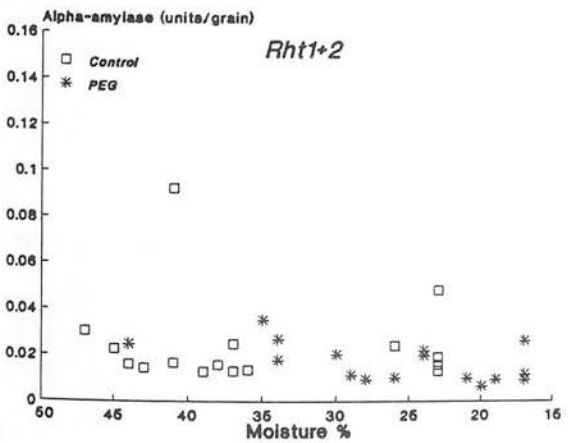


Fig.5.3 Changes in extractable alpha-amylase activity during ear culture on Control (——) or PEG (- - -) media. Individual ear replicates as follows: □=Control; * = PEG.









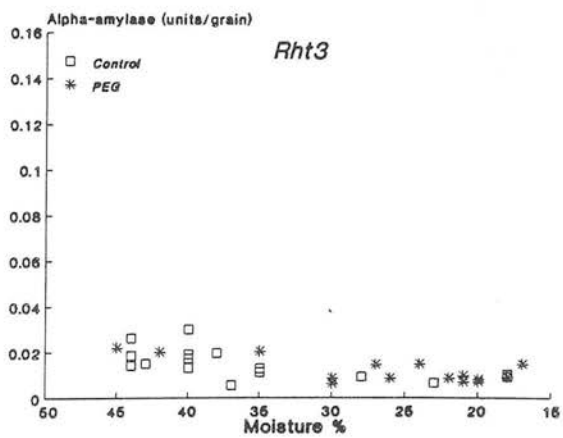
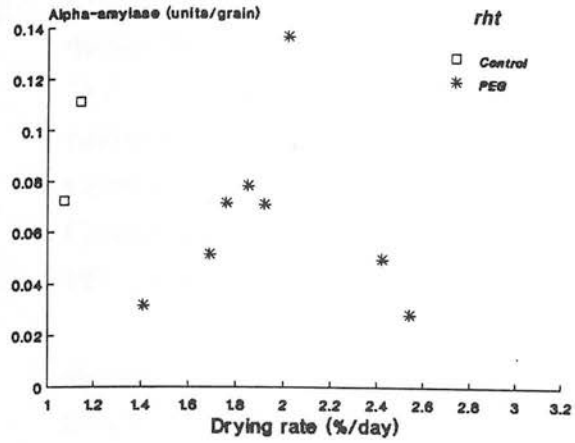
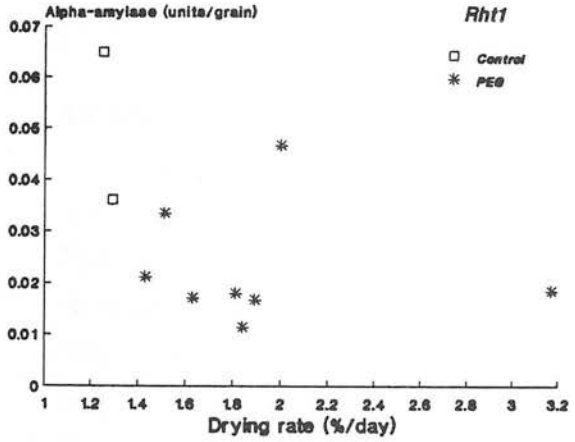
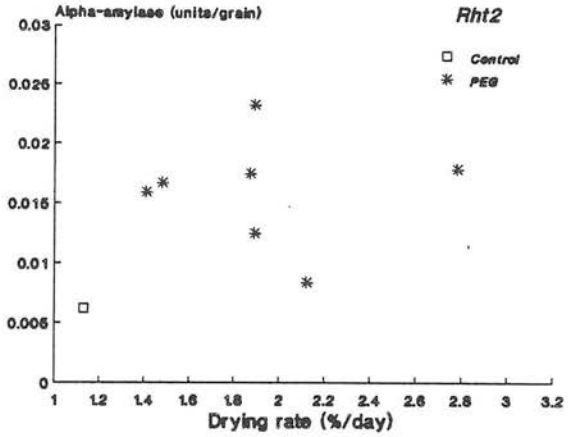
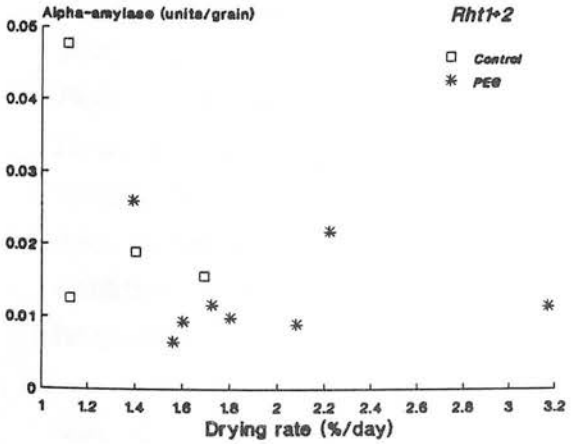


Fig.5.4 Relationship between moisture percentage and extractable alpha-amylase activity of individual ears cultured on Control (□) or PEG (*) media.









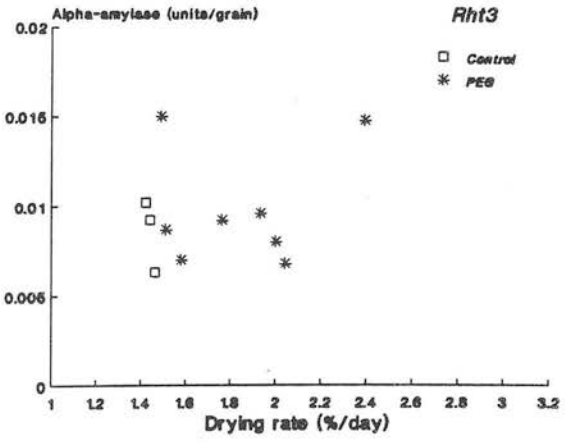


Fig.5.5 Relationship between drying rate and extractable alpha-amylase activity of individual ears having dried to 25% moisture or less. (Control=□; PEG =*).

Control samples were between 30-50% moisture, while PEG samples were mostly below 30%. In *Rht3*, however, the final Control samples had dried to 18% moisture and had alpha-amylase activity as low as that found in PEG-cultured samples. In *Rht2*, between 40% and 30% moisture, there are four Control ears with rather higher alpha-amylase activity than PEG or other Control ears. For the other lines, there are too few examples of Control and PEG samples at a similar moisture level to allow many comparisons.

The difference between mean moisture percentage at D0 and measured moisture percentage at time of sampling was used to estimate the drying rate of all ears of 25% moisture or less. This was then plotted against alpha-amylase activity (Fig.5.5). The scatter graphs (note different scales) show that there was no relationship between drying rate and alpha-amylase activity in any of the five genotypes.

5.4 Discussion.

Ear culture with PEG does appear to be a suitable system in which to manipulate grain drying rate. Earlier experiments found that concentrations of PEG greater than 50g/l produced qualitatively similar results, but were difficult to work with due to the viscous nature of the solutions. Solutions weaker than 50g/l were not tested, but it is likely that a range of concentrations could be used to produce faster or slower grain drying rates. PEG has been widely used as an osmotic medium in plant growth and germination studies. Although some toxic effects have been reported (Lagerwerff, 1961), dialysis or ion exchange successfully removes the heavy metal impurities responsible. Lawlor (1969, 1970) reported that, in plants with damaged root systems, high molecular weight PEG (>1000 MW) entered the plant, leading to reduced water absorption by roots, and dessication of plants, probably by blocking the pathway of water movement. In undamaged root systems only very small amounts of PEG entered the plant. In the present system, entry of PEG into the ear would be unlikely to have any damaging effects. Any enhancement of desiccation could be considered beneficial.

Microbial contamination was a problem in the Control media by the later stages of the experiment, but PEG media appeared to be unaffected. Although contamination was recognised as a potential problem, it was thought that the cooled water bath would minimise the growth of micro-organisms. Similar precautions have been successful in most of the reported studies of ear culture (Donovan and Lee, 1977; Lee *et al*, 1989). However, most of these

studies have been with younger ears which possibly have fewer surface micro-organisms than older material. In germination tests, and half-grain incubation studies, it has been found that mature grains have greater microbial contamination and require more stringent sterilisation than younger grains (Armstrong *et al*, 1982). Cervantes et al (1989b), found that addition of an antibiotic, nystatin (0.05%), to the culture medium prevented growth of micro-organisms and maintained the pH of the media, without the need for sterilization of the culture medium, or a cooled water bath. Their study was with relatively young (15 DAA) ears, and it remains to be seen whether the method is successful for older material.

There is only one reported study of the use of PEG in ear culture. However, it is not strictly comparable with the present work. (Caley, 1986) used much younger ears (25 DAA) in her study of the termination of grain growth. PEG appeared to cause premature onset of water loss and grain ripening, which resulted in the sudden curtailment of grain growth. The intention in the present experiment was to begin ear culture after maximum dry weight had been reached, so that the treatment would only affect water content and drying rate. However, it is clear from the results that dry weight continued to increase in the Control ears and also in some of the PEG ears. Final dry weight was greater than in the equivalent field-dried grains (3.3.1), but so too was the initial grain dry weight, indicating that the cultured ears were not a representative sample of the field plot. This was no doubt due to the (intended) selection of large, still-greenish, uniform ears for the culture experiment, at a time when premature ripening was affecting the field plots. In fact, between D1-18 of the culture experiment, (44-61 DAA in the field trial), dry weight of the field samples actually fell. As previously discussed, (2.4.1) this may not be a true loss of dry weight, rather a reflection of the variability in the field plots. The higher grain dry weight of the cultured ears - ranging from 13.17mg (*Rht1*) to 20.3 mg (*Rht1*) heavier than the equivalent field samples- reflects the difference between potential and actual yield, and illustrates the yield-reducing effect of premature ripening. The differences between the lines in dry weight increase during culture may reflect initial differences in physiological development, and thus different positions on the curve/plateau of dry weight increase. Ears of the same "age" - 40 "DAA"- were carefully selected at the start of the experiment, but greater uniformity could have been attained if ears had been labelled at anthesis, and later selected by "age" (to account for possible differences in the rate of development after anthesis). An alternative explanation for the

differences between the lines is suggested by the fact that it was *Rht3* and *Rht1+2* which gained the most dry weight during culture. The *Rht3* gene has been associated with reduced grain weight, thought to be due to higher grain number, and therefore increased competition for assimilates (Flintham and Gale, 1983). *Rht3* had the lightest grains at the start of culture, and it is possible that the culture medium supplied a higher level of assimilates than the intact plant, thereby altering source-sink relationships.

PEG in the culture medium had a significant effect on the water content of caryopses. The glumes, lemmas and paleas of ears cultured on PEG were visibly drier. Water content of external grain structures was not measured in the present experiment, but an earlier study (not shown) showed that, after 6 days of culture, the moisture percentage of glumes + lemmas + paleas was 6.9%, 9.1%, and 62.7%, for ears cultured on 100g/l PEG, 50g/l PEG, or Control media respectively. The equivalent figure for intact plants was 58%, indicating that the effect was a reduction of water in the PEG ears rather than an increase in the Control ears. Barlow *et al*, (1983) cultured immature wheat ears in media of varying sucrose concentration and found that, while water potential of bracts was reduced, the water and osmotic potentials of the grain were unaffected by increasing sucrose concentration. This is in agreement with studies indicating that developing grains appear to be able to withstand drought by regulating water loss (Barlow *et al.*,1980; Brooks *et al.*, 1982). It seems likely that in older grains, having reached maximum dry weight, water loss is no longer restricted. Therefore the drying of external floral structures would set up a water potential gradient that would tend to increase the flow of water from the grain and this may explain the effect of PEG on grain water content. However, Lee and Atkey (1983) found that the removal of the lemma and palea did not appear to affect the rate of water loss from recently-excised (15-30 min) caryopses. The age of the grains used in their study is not known; if they are younger grains then the result is in agreement with those of Barlow *et al* (1980; 1983) discussed above. If, on the other hand, they were mature, drying grains, it might be that the bracts had not dried out enough, in the relatively short time between grain excision and bract removal, to allow the development of the envisaged water potential gradient.

The present study showed that ear culture on PEG was successful in increasing grain drying rate. However, whether this was associated with lower alpha-amylase activity is less clear. Comparisons between the treatments at any one sample day could be affected by the moisture content and /or "age" of

individual ears. For instance, at D14, the mean moisture percentage of Control ears was 36% while that of PEG ears was 21%. Corresponding "ages" were 44-47 "DAA" and 51+ "DAA" for the Control and PEG ears. To some extent, comparing treatments at a particular moisture level represents a comparison of drying rates. That is, it is likely that Control samples will have taken longer than PEG samples to dry to a particular moisture level. However, such comparisons are limited by the number of ears from each treatment, and are biased towards Control samples at higher moisture levels, and towards PEG samples at lower levels. Ideally, the culture of the Control ears should have been continued until they had dried down to a similar moisture content as the PEG ears. This would have allowed a better comparison of alpha-amylase activity in harvest-ripe grains having dried at different rates. In the absence of harvest-ripe Control ears, drying rate and alpha-amylase were compared in a sample of ears of 25% moisture or less. The figure of 25% was a fairly arbitrary choice and it is possible that a different sample of ears might have produced a different result. It is assumed that any effect of PEG on alpha-amylase activity is via its effect on drying rate and that there is no direct effect. Therefore, the fact that the sample chosen was biased towards PEG ears should not affect the relationship. It is interesting to note the range in drying rate (Fig 5.5, X-axis) even among PEG samples. This reflects the variation in moisture percentage observed between the replicate ears at each sample day. The variation was surprising, given the fairly uniform D0 moisture percentage (47.3%±2.02(SE), 47.2%±1.03, 48.6%±1.6, 48.1%±1.51, and 46.5%±0.26 for *rht, Rht1, Rht2, Rht3*, and *Rht1+2* respectively), and the controlled environmental conditions, and suggests variation in grain and/or ear factors involved in water loss. Such factors might include variation in the rate of the deposition of lipids in the chalazal zone (Sofield *et al.*, 1977), extent of blockage of xylem elements by pectic substances (Cochrane, 1985), or numbers of stomata in the pericarp (Cochrane, 1983). Despite the range of drying rates however, there was no relationship with alpha-amylase activity. It has already been suggested (section 2.4.4) that any relationship between alpha-amylase activity and drying rate may not be apparent at relatively fast drying rates, and it is possible that the individual ears shown in Fig. 5.5 all dried too quickly to have any effect on alpha-amylase activity. The slowest drying rate was 1.07 %/day for an *rht* ear, compared to the 0.6 %/day "slow" drying rate found by Gale et al. (1983) to increase alpha-amylase activity. Extending the culture period of Control ears would have resulted in a sample of ears with slower drying rates. However,

increasing the duration of culture would exacerbate the problem of microbial contamination, discussed above. It might be better to culture older ears for a shorter period of time. If for example, 44-47 "DAA" ears (approximately 40-35% moisture) were used they might dry sufficiently before contamination became a problem.

This study has shown that PEG can be succesfully used to alter grain drying rates. The method lends itself to more detailed investigations of grain water relations and drying rate than in the present work. For example, by transferring ears between Control and PEG media the effects of short periods of water stress could be investigated, and, if drying rate does influence alpha-amylase activity, the critical period could be identified. It is thought that it is the rate of drying between 40% and 20% moisture that is important (Gale *et al.*,1983; Kettlewell and Astbury, 1990) but it may be that grains are at risk for only part of this period.

6. Variation in alpha-amylase activity within ears: effects of moisture and growth regulator-sensitivity.

6.1. Introduction.

The variation in grain alpha-amylase activity within and between ears of Fenman has been discussed (section 2.4.5). The studies reported here were designed to investigate the reasons for the variation between individual grains, with the hope of obtaining information relating to the mechanisms involved in the control of PMAA in Fenman.

Gale *et al.* (1987) have suggested that within-ear variation in alpha-amylase activity is related to position on the ear. They found that, in field-grown Maris Huntsman, grains in the lower-central region of the ear, particularly in floret position b, contained most activity. It was suggested that such grains would have had a slower drying rate because, a) they were older than other grains, and b) they would be slower to dry after wetting than distal floret positions. Evers and Ferguson (1980) found a somewhat similar pattern in some, but not all, ears of Maris Huntsman. This basic pattern was masked in glasshouse-grown ears, which contained grains of abnormally high activity in no discernable pattern. In neither field-grown, nor glasshouse-grown ears was alpha-amylase activity related to grain weight. Cornford and Black (1985) found that the distribution of alpha-amylase activity within ears of Fenman was apparently random, although they suggested that the grains with high activity may have had higher than the average water content. A subsequent experiment, however, failed to show a relationship between alpha-amylase activity and water content, among grain samples from the same ear (Cornford *et al.*, 1987). Alpha-amylase activity is known to vary considerably between grains on the same ear, although the pattern of water content appears to be predictable, and related to grain position. What is needed therefore, is a non-destructive method of determining moisture content of individual grains which allows subsequent extraction and assay of alpha-amylase. Hopefully, this would lead to an unequivocal answer to the question of whether high alpha-amylase activity is associated with relatively high water content. Most methods of moisture determination are based on bulk samples (Hunt and Pixton, 1974). However, there is considerable interest in the development of methods suitable for individual grains. Kandala *et al.* (1989) used measurements of capacitance, dissipation factor and phase angle to estimate the moisture content in single maize kernels. Kovadlo (1982) produced a sensor with a needle electrode

which allowed capacitance measurements on individual pea seeds during ripening. Near infra-red transmittance methods have also been used (Finney and Norris, 1978). Chambers *et al.* (1989), described the use of nuclear magnetic resonance (NMR) to investigate the relationship between pesticide residues and moisture content in wheat grains. This latter method appeared the most readily applicable to the present study, not least because of the availability of a similar spectrometer. Freeze drying would also appear to be a possible means of estimating grain water content in a non-destructive manner. Thus, in the first experiment reported here, NMR and freeze-drying were investigated as possible methods of non-destructive moisture determination, in order that alpha-amylase activity could be assayed in grains of known moisture content.

The second experiment investigates the possibility that the variability in alpha-amylase activity found in Fenman could be attributable to differences in sensitivity to growth regulators among individual grains. Between varieties, differences in GA-sensitivity of half-grains do not appear to be related to susceptibility to PMAA (section 2.3.9), but the same study also found that some replicates of Fenman appeared not to respond to the growth regulator. Also, it is not known whether alpha-amylase activity detected in replicates incubated without GA_3 was due to enzyme present at the start of the experiment, or was produced during incubation. Finally, there is no information on ABA-sensitivity of developing grains of Fenman. In the previously reported study (2.3.8) there was an indication that high activity grains tended to be located in the same ear/s (see Appendix 2 for full details). This suggests that whatever the "trigger" inducing alpha-amylase production in a particular grain may be, the same factor also affects other grains on the same ear. Thus, in the study reported here, it was hoped to relate PMAA in non-incubated grains to differences in growth regulator sensitivity in grains from the same ear. However, even within "high-activity" ears there is considerable variation between grains. Therefore, in order to obtain as much information as possible from single grains, de-embryonated endosperm slices were halved and each half incubated in different solutions.

6.2 Material and Methods

6.2.1. Plant Material.

Glasshouse grown plants were used for the preliminary NMR investigations. Winter wheat cvs. Fenman and Huntsman were grown by the Crop Production Unit, Scottish Agricultural College. Six plants per pot were grown in Levingtons compost in 20cm diameter pots. Natural light was

supplemented to give an 18h photoperiod, with approximate temperatures of 20/12°C. Plants were watered daily, and fed with liquid feed regularly, and treated for insect and fungal infections as necessary.

Field grown ears were used for the freeze-drying study and the growth-regulator experiment. A single plot (22m x 2m) of Fenman winter wheat was drilled on 10th October 1990, in loam soil at Fulford Camp field, part of the Bush Cereal Trials Centre. Previous cropping was winter barley and winter preceded by winter wheat (3 years). Seed rate was 200 kg/ha and 72 kg/ha each of P_2O_5 and K_2O were applied on 23/10/90. Top dressings of 50, 80, and 80 kg/ha N were applied on 12/3/91, 11/4/91 and 1/5/91 respectively. Weed and disease control was as follows:

Herbicides:	Panther	23/10/90
	Starane	27/5/91
Fungicides	Dorin + Sportak	29/4/91
	Sportak + Patrol	5/6/91
	Tilt Turbo + Bravo + Bavistin	28/6/91

6.2.2. NMR moisture determination

The method of Chambers *et al.*, (1989) was followed. NMR spectra of individual grains of Maris Huntsman and Fenman (aproximately 47-51+ "DAA") were recorded on a Varian EM 360 60 MHz proton spectrometer. Spectrometer control settings were: sweep width 20 ppm, offset -6 , RF power 0.4 mG, scan time 1 min, filter 1, spectrum amplitude 10x12.

6.2.3 Freeze-drier moisture determination.

Twenty ears of Fenman were harvested on 4/9/91 (66 DAA). Individual grains from four ears were removed, weighed, and placed in wells of "microtitre" plates. They were pre-frozen and then freeze-dried (Edwards Model 12K Supermodulyo) to constant weight. As a comparison, a bulk sample of grains from the remaining ears was dividing into six replicates, three of which were freeze-dried as above, while the other three were oven-dried at 80°C. Each individual freeze-dried grain was homogenized in 4 ml buffer (section 2.2.4), and extraction and assay of alpha-amylase activity carried out as previously described (section 2.2.4). Some grains were assayed for fungal alpha-amylase activity. The assay method was exactly the same as that previously described (2.2) except that the pH of the extract was adjusted to 5.0, and the substrate

mixture contained blocked ρ–nitrophenol maltosaccharide (MegaZyme (Aust) Pty. Ltd.) with 5 rather than 7 glucose residues. The shorter chain molecule is less readily attacked by cereal than fungal alpha-amylase, and the ratio of measured activity compared to the standard assay was therefore used as an indication of cereal or fungal origin.

6.2.4. Sensitivity of individual grains to growth regulators.

From 42-57 DAA (approximately37-51 "DAA") 4 ears were sampled daily from the field trial. Fifteen grains from the central spikelets of each ear were removed and surface sterilised with $AgNO_3$ (1%) for 2 min, followed by NaCl (1%) for two min, and several rinses with sterile distilled water. The embryo and distal ends of the grain were removed with a flame-sterilised scalpel, and the resulting endosperm slices were soaked in sodium hypochlorite (1% available chlorine) for 20 min. Each slice was cut in half along the line of the crease and added to buffer solution (1.5ml) in sterile 2ml micro centrifuge tubes. Buffer solution was as previously described (section 2.2), with the addition of either GA_3 (10^{-5}M) and/or ABA (50μM). Solutions were sterilized by filtration, and all work was carried out in a laminar flow cabinet. Each grain was randomly allocated to one of fifteen paired treatments as follows: (1 and 2 represent two halves of grains A...O)

	Endosperm "Half"	
Grain	1	2
A	Unincubated	Unincubated
B	Unincubated	Buffer + GA_3
C	Unincubated	Buffer + ABA
D	Unincubated	Buffer + GA_3 + ABA
E	Unincubated	Buffer
F	Buffer + GA_3	Buffer + GA_3
G	Buffer + GA_3	Buffer + ABA
H	Buffer + GA_3	Buffer + GA_3 + ABA
I	Buffer + GA_3	Buffer
J	Buffer + ABA	Buffer + ABA
K	Buffer + ABA	Buffer + GA_3 + ABA
L	Buffer + ABA	Buffer
M	Buffer + GA_3 + ABA	Buffer + GA_3 + ABA
N	Buffer + GA_3 + ABA	Buffer
O	Buffer	Buffer

Unincubated halves were frozen immediately after sectioning. The rest of the samples were incubated in a shaking water bath (25°C) for 72 h. At the end of

the incubation period, they were frozen for later assay of alpha-amylase activity (2.2.2). Moisture content of grains used in the incubations was estimated from measurements made on additional ears sampled each day. A single grain from the central region of the ear was removed from each of 15 ears. Grains were bulked, divided into three replicates of 5, weighed and oven-dried as described previously (section 2.2.2).

6.3 Results

6.3.1. Moisture content and alpha-amylase activity of individual grains.

a) NMR

Two main problems were encountered. Firstly, the majority of grains were too large to fit into the narrow, glass-walled sample tube. Secondly, it was found that the spectrum obtained for a particular grain was greatly affected by the vertical positioning of the sample tube. An example is shown in Fig. 6.1. Scans a, b, and c were of the same grain of Maris Huntsman and differed only in sample tube position.

b) Freeze-drying.

Grain samples of Fenman and Avalon were dried in the oven at 80°C and in the freeze-drier as a comparison. Samples in the oven had dried to more or less constant weight (<0.0075%/day loss) after 12 days, while freeze-dried samples were still losing water (0.05%/day) after 19 days. The calculated moisture percentages (±SE) were as follows:

	Oven	Freeze-drier
Fenman	17.75 ±0.13%	13.2 ±0.09%
Avalon	18.96 ±0.75%	12.86 ±0.11%

Individual grains from four harvest-ripe ears of Fenman (and two of Avalon) were freeze dried but lack of time meant that alpha-amylase activity was determined for one ear of Fenman only. Mean percentage moisture was 16.4% (Table 6.1). The three basal spikelets were the wettest, but apart from that there was no trend relating grain moisture to position on the ear. Overall, grains from a, b, and c florets had similar mean moisture levels. Of the two shrivelled grains, one (4b) had slightly lower, and one (10c) slightly higher than the average moisture level.

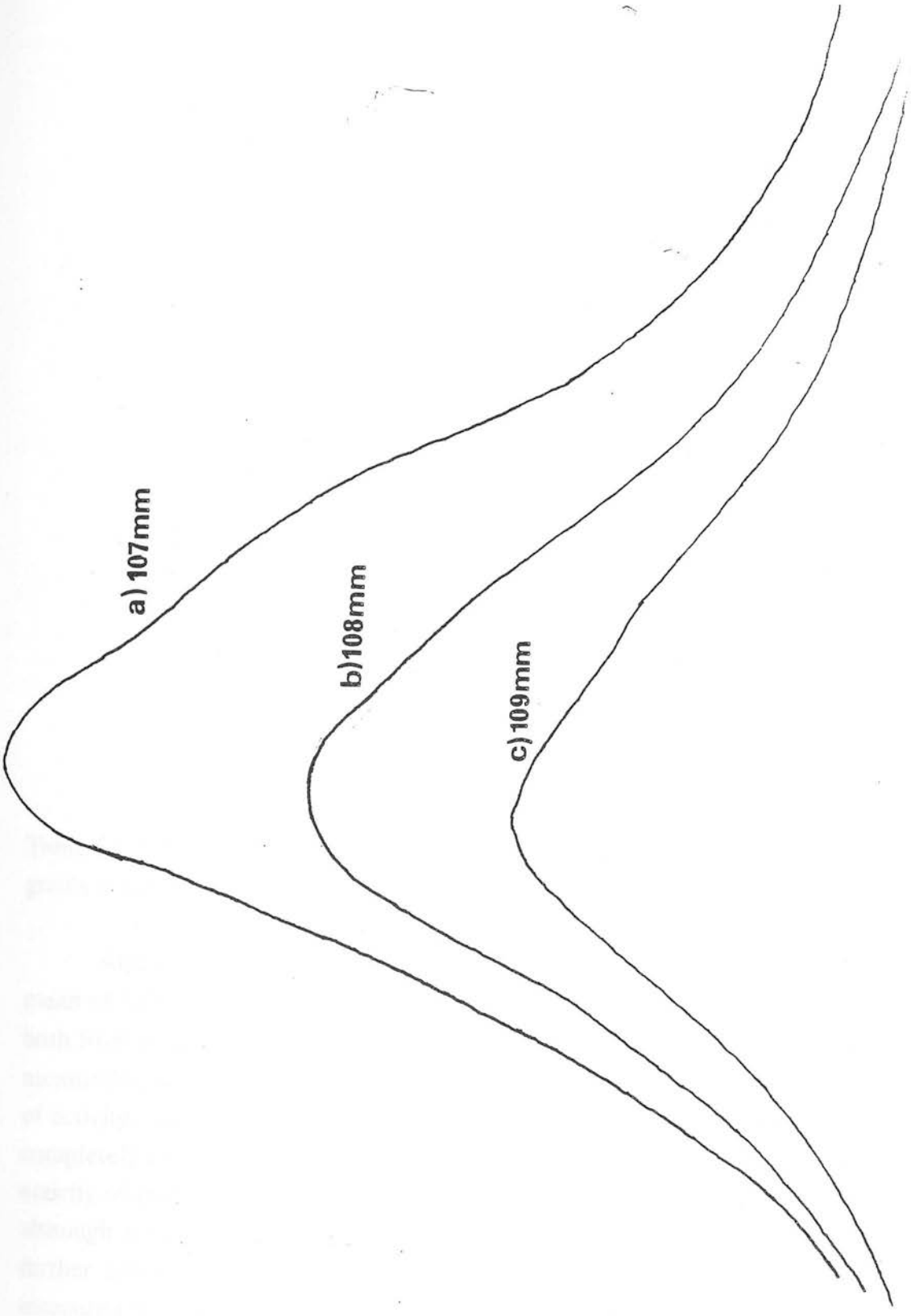


Fig. 6.1. NMR scans of a single grain of Maris Huntsman at different vertical positions within the spectrometer.

	floret					
Spikelet	a	b	c	d	e	mean
1	27.2	21.7	19.1			22.7
2	22.8	17.8	19.4			20.0
3		16.1	22.5	18.0		18.9
4	14.9	15.5	14.0	13.3		14.4
5	18.5	15.4				17.0
6	15.1	16.4	19.2	14.2	16.6	16.3
7	15.7		14.7			15.2
8	14.0	18.4	16.0			16.1
9	18.0		16.1	14.5		16.2
10	15.5	14.5	17.7	15.5		15.8
11	16.0		15.7	13.1		14.9
12	15.7	19.0	15.0	16.5		16.6
13	15.0		16.5	15.0		15.5
14	15.9	14.4	16.9	16.2		15.9
15	14.7		14.0			14.4
16						
17	18.4		13.6			16.0
18	14.0	16.7	15.7			15.5
19	14.8					14.8
mean	16.8	16.9	16.6	15.1	16.6	16.4

Table 6.1. Percentage moisture, determined by freeze-drying, of individual grains within an ear of Fenman.

Alpha-amylase activity per grain ranged from zero to 2.791 units, with a mean of 0.062 units (Table 6.2). The two grains with the highest activity were both from spikelet 14, but this spikelet also contained one of the grains with no measurable activity. Neither of the shrivelled grains had particularly high levels of activity. Spikelet 16 was not included in any measurements because it was completely dried up and mouldy. It was thought possible therefore that the high activity of grains from the adjacent spikelet 14 might be due to fungal amylase, although none of the grains showed visible fungal colonisation. However, further investigation of these, and other random grains, indicated that the measured activity was due entirely to cereal alpha-amylase. Alpha-amylase data was also calculated on a dry weight basis (not shown) but the findings were essentially the same. The shrivelled grains, of lower dry weight, did not have particularly high levels of activity.

	floret					
Spikelet	a	b	c	d	e	mean
1	.007	.030	.002			.013
2	.008	.051	.025			.028
3		.023	.031	.006		.020
4	.065	.006	.037	.004		.028
5	.032	.012				.022
6	.020	.020	.009	.021	0	.014
7	.016		.003			.010
8	.028	.008	.014			.017
9	.032		.012	.002		.015
10	.005	.006	.002	.001		.004
11	.016		.005	.003		.008
12	.014	.008	.002	.001		.006
13	.019		.170	.001		.063
14	2.791	.010	.581	0		.846
15	.001		.001			.001
16						
17	.019		.005			.012
18	.002	.027	.004			.011
19	.003					.003
mean	.181	.018	.056	.004	0	.062

Table 6.2. Alpha-amylase activity (units/grain) of individual grains within an ear of Fenman.

6.3.2. Sensitivity of individual grains to growth regulators.

Incubations were carried out daily between 42-57 DAA, in the hope that this 15 day period would encompass the stage at which GA-sensitivity became apparent in the whole population. When initial assays of the frozen samples indicated little evidence of GA-sensitivity at 49 and 50 DAA it was decided not to assay the earlier samples (in order to save time and money). There was no clear pattern of response to the different incubation treatments. Grains from four separate ears were incubated on each day and selected results (Table 6.3) illustrate a number of points. (Full results for all ears and all days can be seen in Appendix 3).

Ear 1 at 52 DAA (Table 6.3) illustrates the first indication of alpha-amylase activity in unincubated grains (Grain A). Alpha-amylase activity was present in both halves (A1 and A2) of the grain. Of the remaining grains in the same ear a half (L2) incubated in buffer had higher activity than its other half

GRAIN	TREATMENT	DAA / EAR	52 1	53 1	53 4	54 2	55 1	55 2	56 4	57 1
A1	UNINCUBATED		.095	.003	.002	0	.003	.115	1.393	.005
A2	UNINCUBATED		.081	.002	.001	.001	.001	.003	.006	.002
B1	UNINCUBATED		0	.001	.003	.002	.007	.002	1.501	.002
B2	GA_3		.006	.019	.009	.001	.001	.001	.156	.020
C1	UNINCUBATED		.001	.003	.003	.001	.009	.013	.133	.001
C2	ABA		.002	.001	.005	.001	.002	.003	.002	.001
D1	UNINCUBATED		.001	.001	.003	.001	0	.002	.002	.001
D2	GA_3+ABA		.036	.005	.006	.001	.002	.005	.001	.002
E1	UNINCUBATED		0	.033	.002	.001	.002	.004	.001	.023
E2	BUFFER		.003	.002	.004	0	0	.002	.039	.003
F1	GA_3		.079	.524	.029	.117	0	.507	1.825	.001
F2	GA_3		.072	.115	.007	.040	0	.169	.042	.003
G1	GA_3		.139	.386	.416	.088	.002	.095	.157	.002
G2	ABA		.001	.022	.259	.002	.002	.002	.002	.002
H1	GA_3		.166	.400	.068	.132	.002	.004	.662	1.175
H2	GA_3+ABA		.102	.015	.004	.662	.001	.003	.016	.003
I1	GA_3		.225	.111	.900	.036	.001	.002	1.734	.002
I2	BUFFER		.004	.002	.006	.001	.057	.001	.084	.001
J1	ABA		.002	.001	.006	0	.003	.001	.004	.001
J2	ABA		.002	.089	0	.137	.003	.001	.001	.001
K1	ABA		.002	.001	.118	.000	.001	.003	.002	.000
K2	GA_3+ABA		.135	.008	.815	.002	.009	.005	.010	.001
L1	ABA		.009	.006	.002	.165	0	.001	.010	.001
L2	BUFFER		.052	.005	.002	.082	.453	.004	.026	.000
M1	GA_3+ABA		.058	.002	.002	.001	.002	0	.001	.001
M2	GA_3+ABA		.018	.002	.002	.001	.002	.001	.001	.001
N1	GA_3+ABA		.001	.002	.001	.008	.004	.005	.151	.002
N2	BUFFER		.002	.002	.002	.062	.005	.013	.019	.001
O1	BUFFER		.001	.003	.041	0	.001	.001	.052	.000
O2	BUFFER		.002	.003	.005	.001	.002	.002	0	.001
MEANS										
	UNINCUBATED		.030	.007	.002	.001	.004	.023	.506	.006
	BUFFER		.011	.003	.010	.024	.104	.004	.037	.001
	GA_3		.114	.259	.238	.069	.001	.130	.763	.200
	GA_3+ABA		.058	.006	.138	.113	.003	.003	.030	.001
	ABA		.003	.020	.065	.051	.002	.002	.003	.001
	OVERALL		.043	.059	.091	.052	.020	.032	.268	.042

Table 6.3. Selected results showing response (units alpha-amylase activity) of individual grain halves (A1,2....O1,2) to different incubation treatments.

incubated with ABA. Most of the halves incubated with GA_3 had relatively high activity, as did several of the GA_3+ABA halves (D2, H2, K2, M1,. Generally, there was good agreement between both halves of a grain given the same treatment (A, F, J, O).

All of the GA_3-treated halves had high activity in ear 1 at 53 DAA . One of the unincubated halves (E1) had higher activity than other unincubated samples. Samples incubated in buffer only had the lowest level of activity. One half of grain J, incubated in ABA, had relatively high activity while the other half, also incubated in ABA, had low activity. The two halves of grain F, incubated in GA_3, had quantitatively different levels of alpha-amylase activity.

A second ear (ear 4) from 53 DAA had very high levels of activity in some GA_3-treated half grains (I1, G1). No unincubated half had high activity, but two halves in ABA (G2 and K1) did. The other half of both of these grains also had high activity (G1 in GA_3 and K2 in GA_3+ABA). Both halves of grain O were incubated in buffer only, but have different levels of activity.

Ear 2 at 54 DAA had very little activity in unincubated halves, but two halves incubated in buffer only (L2 and N2) had increased levels of activity. The other half of grain L was incubated in ABA and had higher activity than the half in buffer. J2, also incubated in ABA, had relatively high activity, while the other half (J1), given the same treatment, had no measurable activity. The highest activity was in grain H, incubated in GA_3 and GA_3+ABA.

At 55 DAA, ear 1 had little activity in any sample apart from I2 and L2, incubated in buffer only. The other halves of these grains, incubated in GA_3 and ABA respectively, had little or no activity. Ear 2 at 55 DAA showed a high level of alpha-amylase activity in an unincubated half grain (A1), but low activity in the other, also unincubated, half. Relatively high, but quantitatively different, levels of activity were recorded in halves of grain F, both of which were incubated in GA_3.

The highest overall activity was recorded in ear 4 at 56 DAA. There was an apparent asymmetric distribution of activity between similarly-treated halves of grains A, F and O, which were unincubated, incubated with GA_3, and incubated in buffer only, respectively. All of the GA_3-treated halves, except F2, had relatively high activity; some (F1 and I1) had very high activity. Low activity was recorded in all of the ABA and all except one (N1) of the GA+ABA treatments. Almost all of the activity recorded in ear 1 at 57 DAA was due to one half of grain H, incubated in GA_3, with low activity in all other grains and treatments.

6.4 Discussion

Although NMR analysis had appeared to be a promising method of analysing water content of individual grains, the problem of grain size was unexpected. No such problems of fitting grains into sample tubes were reported by Chambers *et al.*, (1989) who used the same variety (Maris Huntsman) in their study. They do however, note that vertical positioning of grains was "surprisingly critical", and suggested adjustment of each grain until the maximum signal was obtained. The present results indicate that differences due to errors in grain positioning were likely to be greater than those due to water content. It was decided that the technique was unlikely to be sensitive enough for the requirements of the project and no more time was spent on it.

There were disparities in the moisture content determined by oven-drying and freeze-drying. That is, more weight was lost in the oven than in the freeze-drier. The process of freeze-drying involves sublimation, under vacuum, of ice-crystals from pre-frozen material. It is therefore unlikely that anything but water would be lost during the freeze-drying process, whereas oven-drying could possibly involve the loss of volatile compounds. However, such loss is reported to be insignificant (Slavik, 1974), and a considerably higher temperature (130°C) than that used in the present study is in fact recommended for determining whole grain moisture content (ASAE, 1978). A more likely explanation for the difference between freeze-dried and oven-dried samples is suggested by the fact that sublimation only occurs if heat is supplied to the frozen material, and while room heat is usually sufficient it may not have allowed loss of all water in the present study. Also, the small size of the grains means that they may have partly thawed before the operating vapour pressure was obtained. The samples were placed on pre-frozen metal trays to try and avoid this. Although the results obtained from freeze-drying may not therefore be comparable with oven-dried samples, it is assumed that individual grains in the freeze-drier do not lose water at a significantly greater or lesser rate than each other. This may not be valid, and could be tested by drying grains previously equilibrated to known (approximate) moisture contents. It was concluded that freeze-drying, although not providing an accurate measure of absolute water content, was suitable for relative comparisons between samples dried in the same way. The method was therefore used to investigate the relationship between moisture content and alpha-amylase activity.

The results from one ear are obviously insufficient to allow firm conclusions about the existence of such a relationship. The most that can be

said is that there is no indication that alpha-amylase activity observed in individual grains of Fenman is related to moisture content, shrivelled grain, or fungal infection. However, there is no way of knowing the previous history of the grains; in particular, the drying rate that resulted in the measured moisture percentage is unknown. While it may be thought that moisture percentage gives an indication of drying rate - ie grains with highest moisture having dried the slowest - it may be that such grains commenced drying later, and the actual drying rate was the same. Measurements of water content of individual grains from different positions on the ear throughout grain development and ripening would help to determine if grains do dry at significantly different rates.

It has been noted (above, and section 2.4) that grains of high activity tended to be found on particular ears rather than completely randomly throughout the population. The present study indicates that within such ears, particular spikelets contain more than one grain with high activity, eg spikelet 14. It should be noted however, that this spikelet also contained a grain with no measurable activity. In the only other study relating alpha-amylase activity of individual ears to spikelet and floret position, Cornford and Black (1985) showed a greater range in activity and more spikelets containing grains of high activity than in the present study. More studies are required to determine the frequence of "high activity" spikelets before a possible explanation can be suggested.

The results of the incubated grain studies are extremely difficult to interpret. In planning the experiment it was anticipated that, as previously discussed (section 2.4), PMAA would increase over a fairly sharply defined time period. Results from 1989 and 1990 indicated that physiological "age" at this stage was about 47±3 "DAA", and sampling for the present experiment began prior to that stage, at about 37 "DAA". Sample size in 1989 and 1990 was ten ears, and it was therefore estimated that at least one in ten ears would show signs of PMAA. As already discussed, it was also thought likely that more than one grain on a particular ear would show PMAA. However, from the 60 ears incubated over the duration of the experiment, 32 were assayed for alpha-amylase activity, and only one (ear 4, 56 DAA) showed high alpha-amylase activity in more than one unincubated grain. It was expected that there would be more such ears, and that the response of the other grains on the ear would give an indication of the GA- and ABA-sensitivity of grains affected by PMAA. If that one ear is representative of PMAA-affected ears it demonstrates a number of interesting observations. Firstly, the asymmetric distribution of

alpha-amylase activity within a grain, most marked in grain A, but also apparent in grains F and O. Both halves of grain F were incubated in GA_3 so the result indicates either a difference in pre-existing alpha-amylase (as in grain A) and/or an asymmetric response to GA_3. Cornford *et al.* (1987) demonstrated that alpha-amylase activity within individual grains of Fenman was associated with aleurone tissue in the ventral portion of the grain. There is no indication from their photographs of differences between median longitudinal sections of the same grain. Further, there are no reports of variation in GA-sensitivity between different grain portions, although Bewley and Black (1983) do note that the assumption that all cells in an isolated aleurone layer are more or less synchronous in their GA-response might not be valid. A second observation from ear 4, 56 DAA, is that no ABA-treated grain half has high activity, and only one (N2) GA_3 + ABA-treated half has increased activity. GA_3 induced a 9-fold increase (compared to untreated control) in alpha-amylase activity in incubated wheat grains (48 DAA), but GA_3 + ABA reduced activity to levels similar to those of untreated grains (King, 1976). Similarly, ABA reduced alpha-amylase activity in isolated wheat aleurone tissue (25 DAA) compared to untreated tissue, while GA_3 + ABA treatment reduced alpha-amylase activity to about 3% of that found in GA_3 alone (Napier *et al.*, 1989). Garcia-Maya *et al.* (1990), found that synthesis of low pI wheat alpha-amylase is insensitive to ABA (in both embryos and aleurone cells), possibly due to pre-existing mRNA transcripts, and that low pI isozymes appear to be independent of GA_3. Together, these results suggest that, in the present work, the majority of alpha-amylase activity in ABA- and GA_3 + ABA-treated grains is due to pre-existing enzyme, but that some activity may be due to newly-synthesised low pI isozymes. Isoelectric focussing would help to answer this question.

Other ears, apart from ear 4, 56 DAA, do show high activity, particularly in GA_3-treated grains, but there is no way of knowing whether the measured activity represents pre-existing PMAA, or was produced during incubation. When all of the GA_3-treated halves show relatively high activity and other treatments very little (eg ear 1, 53 DAA) it seems likely that the enzyme is newly-synthesised. In other ears (eg ear 2, 55 DAA), alpha-amylase activity is evident in some but not all GA_3-treated grains. This indicates either pre-existing PMAA, or variation in GA-sensitivity between grains on the same ear. The acquisition of GA-sensitivity may be a development-dependent process and as such, variation between grains may be related to "age" differences. It was hoped that by using only grains from b and c florets of the central spikelets such

differences would not be significant. If GA-sensitivity is not dependent on "age" but rather, as Black *et al.* (1982) suggested, on the attainment of a critical water level, differences in moisture content between grains may explain the results. In the present study there appeared to be no difference in percentage moisture between a and b florets, but these results were from a single ear and hence may not be representative of all ears. Also, those measurements were made at harvest ripeness, and the relationship between a and b florets may have been different in the younger ears used in the incubation studies. Jenner (1982) found that, in central spikelet positions, b grains had higher water content than a grains. However, this was at 20 DAA, during the linear period of dry matter accumulation and is again, therefore, probably not comparable with the ears used in the present study. In considering apparent variation in GA-sensitivity it is interesting to note that in a previous experiment (section 2.3) it was found that some replicates of Fenman appeared not to respond to GA_3 even at 66 DAA (51+ "DAA" and aproximately 20-24% moisture). It is possible therefore, that in Fenman at least, GA-sensitivity is neither "age" nor moisture dependent.

Alpha-amylase activity was present in some grain halves incubated in buffer only. In ear 4, 56 DAA, such activity would appear to represent pre-existing PMAA. In ear 2, 54 DAA, both halves of grain L, incubated in ABA, and buffer-only, contained alpha-amylase activity, again indicating PMAA. However, in ear 1, 55 DAA, grain L only showed alpha-amylase activity in the buffer-treated half, another indication of asymmetric distribution of PMAA. There seems little reason to suggest that incubation in buffer only would initiate alpha-amylase production, although Cornford and Black (1985) found that, in some wheat cultivars, immature (25 DAA) half-grains given a 72h pre-incubation in buffer subsequently produced alpha-amylase in the absence of GA_3 (though at lower levels than in the presence of GA_3). This could possibly have been low pI alpha-amylase, for the reasons discussed above.

It is difficult to draw conclusions from these two studies of individual grains. In the first experiment, there appeared to be no relationship between alpha-amylase activity and moisture content, shrivelled grain, or fungal infection, but this was based on results from one ear only and obviously needs to be further investigated. In the second experiment, although a greater number of ears were investigated, incidence of PMAA was not as widespread as expected, again allowing few conclusions. However, the study did indicate that the timing of PMAA is not as sharply defined as was indicated by the bulk samples used in previous experiments (section 2.3), and that GA-sensitivity is by no means

uniform either between or within ears.

7. General conclusions and suggestions for future work.

PHS is an environmentally-influenced phenomenon, of sporadic occurrence. As such, it presents particular problems as a research subject. In the present study, the main bulk of the investigations were carried out on field-grown material, and were thus influenced by the prevailing climatic conditions. The summer months of 1989 and 1990 were characterised by above average temperatures and generally below average rainfall. Such conditions are not conducive to PHS. Thus it is not surprising that, except for occasional grains of Maris Huntsman in 1990, there were no observations of PHS during the course of the investigations.

In the absence of PHS the focus of the study was narrowed to concentrate on PMAA, in particular PMAA in the variety Fenman. From the data obtained, albeit in "good" conditions, it appears Fenman is extremely susceptible to PMAA, Maris Huntsman less so, and Avalon, Apollo, Brock and Mission apparently resistant. The reasons for the extreme susceptibility of Fenman are not clear. Although the gene/s causing PMAA in Fenman and Maris Huntsman appear to be derived from the same source - Professeur Marchal - the effect seems to be modified by other factors. Since observed PMAA in Maris Huntsman was much lower than that of Fenman grown under the same conditions, the suggestion is that these other factors are genetic rather than environmental. There may be genetic differences in sensitivity to environmental factors that influence PMAA. That genetic differences in sensitivity to environment do exist was shown by the results of the germination tests, in which genotypes differed in their response to the high germination temperature. It was suggested (Chapter 3) that the Maris Huntsman genotypes, in addition to their differences in GA-sensitivity, also differed in sensitivity to the environmental "trigger" that initiates PMAA. This might explain why the effect of the *Rht* genes in reducing PMAA did not appear to be related to their effect on aleurone sensitivity. If this hypothesis of genetic differences in sensitivity to environment were correct then *Rht3* would be expected to show no response to environmental conditions while *Rht1*, *rht* and Fenman would show increasing sensitivity, such that Fenman would have the highest level of alpha-amylase in a given environment. Testing this hypothesis would be easier were the environmental "trigger" better understood. Although cool, wet conditions and other factors that delay grain drying have been associated with increased PMAA, it seems unlikely that these factors themselves induce alpha-

amylase synthesis. The results from the present study suggest that some grains synthesise PMAA and some do not, although a decision on what is a "normal" level of activity and what is not is rather difficult. In chapter 2 it was suggested that the proportion of grains with alpha-amylase activity greater than 0.1 enzyme units/grain was increased in the wetting and covering treatments relative to the control. However, the results from Chapter 4 suggest that, in warm, dry conditions, the mean level of activity in "normal" grains of Fenman may be as low as 0.018 units. If the individual grain results from Chapter 2 are then re-examined with this as a baseline (Table 7.1) it indicates that the treatments, in addition to their effect on the proportion of affected grains, also increased their level of activity relative to the control:

	$\leq$0.018 units/grain		>0.018 units/grain	
	No.grains	activity/grain	No.grains	activity/grain
Control	15	.009	15	0.269
Wet	12	.009	18	1.711
Covered	7	.009	23	1.22

Table 7.1. Effects of covering and wetting treatments on PMAA in Fenman. (averaged individual grain date from 49-66 DAA.)

In Chapter 4 it was suggested that environmental effects may be mediated in different ways at different stages of development, ie that an earlier environmental "trigger" might affect the proportion of affected grains while their level of activity was determined by later effects such as rate of grain drying. The results summarised above suggest that, with respect to activity levels, the effect of an earlier trigger (ie difference between Control and Covered) is greater than that due to drying rate (ie difference between wet and covered treatments).

How might the early environmental effect change the proportion of affected grains? The studies of individual grains (Chapter 6) indicated that there was variation between and within ears with respect to GA-sensitivity. It would be useful to investigate whether this variation could be influenced by environmental conditions. Similar studies should be repeated using plant material grown in conditions where the factors that may be responsible for the early "trigger" ie high mean temperature, high humidity, large diurnal temperature change can be manipulated.

The results above suggest that effects of grain drying rate on PMAA may be less important than previously thought. The onset of PMAA appears to

occur between about 40 and 30 % moisture. The results presented in Chapter 2 suggest that alpha-amylase activity rises steeply and then levels off. It may be that this is due to reduced moisture levels. If this is so it indicates that the risk period covers a rather narrow range of grain moisture content, possibly explaining why grain drying rates over a wider range were not related to alpha-amylase activity. The key question is: At what moisture level is alpha-amylase synthesis inhibited? This would appear to be quite difficult to investigate experimentally. Isolated aleurone layers are the standard material for examining alpha-amylase synthesis, but are invariably fully hydrated in buffer solutions. The possibility of using controlled conditions to equilibrate isolated aleurone layers to different moisture levels should be investigated. If such a system were succesful it could be used to examine the effects of re-wetting on alpha-amylase synthesis. Such studies would help in determining exactly when the risk period for PMAA is, and if increased moisture levels due to rainfall could re-initiate alpha-amylase production. Ear culture, as was suggested, could be used in a similar way but does not allow direct control of the moisture level in the experimental system.

The apparent levelling of alpha-amylase levels could be due to factors other than reduced moisture. Once alpha-amylase synthesis in germinating grains has begun, it rises rapidly but is apparently inhibited after about 3-4 days of germination. As discussed in Chapter 3, it is possible that sugars are responsible. No relationship was found between alpha-amylase activity and endosperm or embryo sucrose levels in Maris Huntsman, but it is possible that in Fenman, with much higher levels of alpha-amylase, such a control mechanism might exist.

If the risk period is shorter than previously thought, it offers an explanation for how an early environmental "trigger" might affect levels of activity. If PMAA is dependent on GA-sensitivity, an early environmental treatment might cause an earlier onset of GA-sensitivity thus extending the risk period. In this respect it may be significant that, although all genotypes showed GA-sensitivity (indicating that this alone was not responsible for PMAA), in Fenman the onset of responsiveness occurred at a slightly higher moisture level. Thus, if aleurone responsiveness begins at a sufficiently high moisture content, alpha-amylase synthesis may proceed and reach excessive levels. In less susceptible varieties, onset of aleurone responsiveness may occur at moisture levels too low for alpha-amylase synthesis.

In summary then, the proposed model is that an early environmental

"trigger", possibly high humidity/high temperature/large diurnal temperature changes triggers an earlier onset in GA-sensitivity in some grains. The subsequent production of alpha-amylase occurs over a narrow moisture range. The number of grains affected may depend on their stage of development and/or moisture content at the time of the environmental "trigger". Genotypic differences may be based on variation in sensitivity to the environmental "trigger".

This is obviously based on limited data but would appear more satisfactory in explaining the present results than existing theories. It provides a basis for future studies.

Such studies should probably be based on Fenman because its extreme susceptibility to PMAA, even in good weather conditions makes it useful as a research subject. However, it should be noted that Fenman may not be typical of cultivars susceptible to PMAA. In addition, its soft-milling qualities and weak flour strength mean that it is unsuitable for bread-making for reasons quite apart from its high alpha-amylase activity. It would appear sensible to include recognised breadmaking varieties in future studies. To date, PMAA has yet to be unequivocally demonstrated in any of the current breadmaking varieties. Avalon, as the major bread-making wheat of the past decade, was included in the present study for these reasons.

The main reasons for selecting field rather than controlled environment conditions were outlined in section 2.1. Ideally, field-based studies should be of a long-term nature, comparing results from "good", "normal", and "bad" years. It may be possible to reduce the length of studies and obtain extra information by the use of rain simulators and/or rain-out shelters to simulate or prevent rainfall. There are of course, disadvantages to field studies; while they are probably best for time-course studies and genotypic comparisons, the uncontrolled and unpredictable diverse environmental factors mean that they are unlikely to provide sufficient informations on the mechanisms involved. Thus, while the present study has provided detailed and useful information on the timing of PMAA in relation to grain development, further studies will have to be in controlled environments. Ear culture appears to be a useful means of examining environmental effects over short time periods. However, the variation within ears suggests that the development of a method to culture individual grains would be even more useful.

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APPENDIX 1. GRAIN DEVELOPMENT SCALE FOR WHEAT (Riffkin, 1987)

Age (days after anthesis)	Appearance of pericarp	Size of caryopsis (mm)	Size of embryo (mm)	Size of endosperm (mm)	Description of endosperm
1	very pale green crease light green	1.0 x 2.0	-	-	-
2	pale green	1.2 x 2.0	-	-	transparent, liquid
5	pale green	3.0 x 2.5	-	-	transparent, liquid
9	pale green crease green	4.0 x 2.5	-	-	transparent, liquid
12	pale green	5.3 x 3.5	-	4.5 x 3.3	translucent, fluid very fragile
16	pale green	6.0 x 3.5	pin tip	5.5 x 3.5	cream, soft moist contents
19	light-medium green	6.0 x 4.0	1.0 x 1.0	5.8 x 4.0	cream, soft moist contents
23	medium green	6.3 x 4.0	1.8 x 1.2	6.2 x 4.0	white, moist becoming sticky

Age (days after anthesis)	Appearance of pericarp	Size of caryopsis (mm)	Size of embryo (mm)	Size of endosperm (mm)	Description of endosperm
26	green	6.5 x 5.0	2.5 x 2.0	6.0 x 4.5	milk-white, moist-firming
30	green	6.5 x 5.0	2.5 x 2.0	6.0 x 4.8	milk-white, moist dough
33	green-beginning to yellow	6.5 x 5.0	3.0 x 2.0	6.5 x 4.8	milk-white, soft-doughy
37	yellowing-green	6.5 x 5.0	3.0 x 2.0	6.5 x 5.0	pale-cream, sticky dough
40	yellow, crease still green	6.5 x 5.0	3.0 x 2.0	6.5 x 5.0	cream, drying dough
44	yellow-brown pale green crease	6.3 x 4.5	3.0 x 2.0	6.0 x 4.5	cream, firm, imprint partially remains
* 47	mid-brown, slight wrinkling	6.3 x 4.0	3.0 x 2.0	6.0 x 4.0	cream, dry and rubbery imprint remains
* 51	red-brown	6.0 x 3.5	3.0 x 2.0	-	cream, hard and floury becoming brittle

* Caryopsis colour: function of testa pigmentation, i.e. pericarp is transparent

Age (days after anthesis)	Caryopsis - Shape and Tissue Description			Description of embryo	Description endosperm
	Shape	Appearance of transparent (TL) and green (GL) layers	Fused to testa		
1		TL/GL not clearly differentiated	No	-	(Ovule) clear and liquid
2		TL/GL separation just possible, TL light and fluffy	No	-	Clear and liquid
5		TL/GL separate with difficulty, TL fleshy/moist	No	-	Liquid, no determinate shape
9		TL/GL separate with difficulty, TL fleshy-moist, GL green	No	-	Liquid and fragile
12		TL/GL separate cleanly, TL medium moist	No	-	Can be squeezed out, shallow cheeks
16		TL/GL separate cleanly, TL medium moist	No	cream- pin tip	Cheeks beginning to fill, dorsal area thin
19		TL/GL separate easily, TL medium moist	No	cream- fragile	Cheeks filling, dorsal area thickening
23		TL/GL separate easily, TL medium-thin moist	No	cream- moist and soft	Cheeks filled, dorsal area thickened

Age (days after anthesis)	Caryopsis - Shape and Tissue Description			Description of embryo	Description endosperm
	Shape	Appearance of transparent (TL) and green (GL) layers	Fused to testa		
26		TL/GL separate easily, TL becoming thinner	very slightly	Light yellow moist-firming	Cheeks fattening, dorsal area thickened
30	"	TL/GL separate easily, TL still medium, thin, moist	Partial	Light yellow on pale Scutellum, firming	Cheeks and dorsal area well fattened
33	"	TL/GL sticky separation, TL thin, opaque - drying	Partial	Yellow on pale/ cream scutellum, firm	Cheeks and dorsal area fattened
37	"	TL/GL sticky to separate, TL thin - drying	Yes	Yellow on pale/ cream scutellum firm	"
40		TL/GL sticky to separate, TL thin - transparent, drying	Yes	Yellow on pale scutellum, firm-crisp texture	"
44		TL/GL difficult to separate, TL becoming flakey	Yes	Yellow, firm-crisp	Firm texture, thumb-nail imprint partially remains
47	slight wrinkling	TL/GL difficult to separate, TL transparent/flakey	Yes	"	Dry and rubbery, imprint remains
51	shrunken appearance	"	Yes	Firm/oily in texture	Hard-floury, becoming brittle

APPENDIX 2 ALPHA-AMYLASE ACTIVITY OF INDIVIDUAL GRAINS
(See Section 2.3.8)

1. CONTROL

49 DAA EAR	GRAIN A	B	C	EAR TOTAL	EAR MEAN
1	.005	.003	.004	.012	.004
2	.028	.045	.015	.088	.029
3	.034	.019	.043	.096	.032
4	.042	.016	.041	.099	.033
5	.034	.044	.057	.135	.045
6	.043	.011	.083	.137	.046
7	.013	.090	.043	.146	.049
8	.063	.061	.041	.165	.055
9	.059	.037	.093	.189	.063
10	.076	.075	.084	.235	.078
52 DAA					
1	.010	.007	.008	.025	.008
2	.014	.013	.015	.042	.014
3	.006	.025	.015	.046	.015
4	.032	.008	.009	.049	.016
5	.005	.012	.034	.051	.017
6	.030	.040	.015	.085	.028
7	.009	.042	.112	.163	.054
8	.093	.065	.053	.211	.070
9	.047	.080	.088	.215	.072
10	1.026	.039	6.025	7.090	2.363
56 DAA					
1	.006	.005	.007	.018	.006
2	.015	.011	.011	.037	.012
3	.015	.007	.018	.040	.013
4	.035	.006	.004	.045	.015
5	.006	.029	.046	.081	.027
6	.067	.015	.034	.116	.039
7	.007	.173	.042	.222	.074
8	.041	.281	.062	.384	.128
9	.011	1.171	.030	1.212	.404
10	.047	.013	1.504	1.564	.521
59 DAA					
1	.005	.002	.002	.009	.003
2	.004	.002	.006	.012	.004
3	.004	.004	.002	.010	.003
4	.010	.009	.004	.023	.008
5	.024	.007	.007	.038	.013
6	.007	.023	.011	.041	.014
7	.025	.012	.008	.045	.015
8	.031	.044	.058	.133	.044
9	.077	.006	.051	.134	.045
10	.012	.095	.038	.145	.048

63 DAA EAR	GRAIN A	B	C	EAR TOTAL	EAR MEAN
1	.002	.007	.007	.016	.005
2	.014	.008	.013	.035	.012
3	.008	.005	.024	.037	.012
4	.026	.013	.056	.095	.032
5	.055	.025	.046	.126	.042
6	.035	.010	.107	.152	.051
7	.049	.035	.072	.156	.052
8	.068	.091	.048	.207	.069
9	.097	.051	.067	.215	.072
10	12.822	.040	.012	12.874	4.291
66 DAA					
1	.003	.002	.002	.007	.002
2	.003	.002	.003	.008	.003
3	.005	.005	.006	.016	.005
4	.007	.004	.010	.021	.007
5	.008	.008	.006	.022	.007
6	.012	.009	.011	.032	.011
7	.019	.044	.016	.079	.026
8	.018	.059	.005	.082	.027
9	.040	.018	.031	.089	.030
10	.046	.073	.015	.134	.045

2.WET

49 DAA EAR	GRAIN A	B	C	EAR TOTAL	EAR MEAN
1	.020	.021	.017	.058	.019
2	.052	.021	.011	.084	.028
3	.065	.025	.090	.180	.060
4	.169	.160	.078	.407	.136
5	.083	.048	2.484	2.615	.872
6	.008	2.100	1.540	3.648	1.216
7	5.896	.074	2.984	8.954	2.985
8	5.674	1.817	2.908	10.399	3.466
9	2.726	5.411	2.645	10.782	3.594
10	4.765	15.305	2.383	22.453	7.484
52 DAA					
1	.005	.020	.008	.033	.011
2	.013	.036	.009	.058	.019
3	.037	.007	.016	.060	.020
4	.090	.010	.133	.233	.078
5	.267	.007	.019	.293	.098
6	.563	.521	.309	1.393	.464
7	.035	.019	3.962	4.016	1.339
8	.010	.008	8.384	8.402	2.801
9	2.520	9.516	1.220	13.256	4.419
10	6.124	2.853	8.705	17.682	5.894

56 DAA EAR	GRAIN A	B	C	EAR TOTAL	EAR MEAN
1	.002	.002	.002	.006	.002
2	.001	.002	.003	.006	.002
3	.003	.004	.003	.010	.003
4	.002	.002	.007	.011	.004
5	.006	.008	.006	.020	.007
6	.012	.007	.010	.029	.010
7	.005	.029	.025	.059	.020
8	.060	.005	.003	.068	.023
9	.009	.006	.071	.086	.029
10	.083	.019	2.665	2.767	.922
59 DAA					
1	.006	.007	.006	.019	.006
2	.008	.005	.007	.020	.007
3	.022	.010	.004	.036	.012
4	.032	.019	.008	.059	.020
5	.030	.013		.043	.022
6	.046	.013	.010	.069	.023
7	.046	.019	.011	.076	.025
8	.018	.074	.077	.169	.056
9	.028	.940	.078	1.046	.349
10	15.410	.089	2.730	18.229	6.076
63 DAA					
1	.011	.007	.007	.025	.008
2	.026	.006	.007	.039	.013
3	.012	.008	.025	.045	.015
4	.143	.026	.023	.192	.064
5	.089	.048	.078	.215	.072
6	.060	.029	.050	.139	.046
7	.341	.111	.122	.574	.191
8	.025	6.856	.471	7.352	2.451
9	7.956	7.915	.144	16.015	5.338
10	18.563	1.050	.267	19.880	6.627
66 DAA					
1	.009	.004	.006	.019	.006
2	.006	.014	.005	.025	.008
3	.011	.006	.010	.027	.009
4	.013	.010	.014	.037	.012
5	.029	.004	.002	.035	.012
6	.034	.007	.071	.112	.037
7	.018	.006	.091	.115	.038
8	2.009	.029	.009	2.047	.682
9	2.554	5.967	.102	8.623	2.874
10	11.358	.687	6.853	18.898	6.299

3. COVERED

49 DAA EAR	GRAIN A	B	C	EAR TOTAL	EAR MEAN
1	.140	.011	.007	.158	.053
2	.021	.016	.171	.208	.069
3	.080	.032	.143	.255	.085
4	.070	.071	.253	.394	.131
5	.032	.015	.444	.491	.164
6	.018	.414	.094	.526	.175
7	.100	.242	.230	.572	.191
8	.178	.222	.384	.784	.261
9	.193	.828	1.868	2.889	.963
10	2.787	1.747	6.280	10.814	3.605
52 DAA					
1	.002	.011	.001	.014	.005
2	.016	.008	.006	.030	.010
3	.130	.005	.030	.165	.055
4	.106	.044	.030	.180	.060
5	.102	.013	1.151	1.266	.422
6	.057	1.181	.717	1.955	.652
7	.113	.134	1.797	2.044	.681
8	.043	2.312	.082	2.437	.812
9	.004	6.098	.070	6.172	2.057
10	.057	.019	7.320	7.396	2.465
56 DAA					
1	.004	.012	.004	.020	.007
2	.014	.063	.030	.107	.036
3	.086	.074	.028	.188	.063
4	.043	.026	.119	.188	.063
5	.046	.969	.170	1.185	.395
6	4.767	.094	.029	4.890	1.630
7	.035	.038	6.724	6.797	2.266
8	.088	5.967	1.363	7.418	2.473
9	1.605	8.561	3.554	13.720	4.573
10	10.752	3.089	7.431	21.272	7.091
59 DAA					
1	.004	.003	.018	.025	.008
2	.033	.007	.005	.045	.015
3	.052	.005	.013	.070	.023
4	.043	.020	.010	.073	.024
5	.005	.040	.041	.086	.029
6	.022	.019	.068	.109	.036
7	.033	.097	.029	.159	.053
8	.073	.102	.066	.241	.080
9	.059	.017	3.645	3.721	1.240
10	.065	2.645	5.169	7.879	2.626

63 DAA EAR	GRAIN A	B	C	EAR TOTAL	EAR MEAN
1	.006	.014	.005	.025	.008
2	.043	.075	.056	.174	.058
3	.042	.095	.089	.226	.075
4	.040	.157	.055	.252	.084
5	.044	.110	.122	.276	.092
6	.154	.080	.170	.404	.135
7	4.483	.021	.087	4.591	1.530
8	4.018	.425	.410	4.853	1.618
9	.570	7.612	.557	8.739	2.913
10	.085	9.369	17.668	27.122	9.041
66 DAA					
1	.006	.005	.004	.015	.005
2	.007	.008	.003	.018	.006
3	.009	.014	.004	.027	.009
4	.073	.015	.009	.097	.032
5	.026	.047	.032	.105	.035
6		.056	.036	.092	.046
7	.055		.041	.096	.048
8	.010	.145	.028	.183	.061
9	3.614	.127	.220	3.961	1.320
10	5.896	3.826	3.776	13.498	4.499

APPENDIX 3. FULL RESULTS OF INCUBATIONS OF INDIVIDUAL GRAINS (See Section 6.3.2)

49 DAA		EAR			
	TREATMENT	1	2	3	4
A1	UNINCUBATED	.004	.001	.002	.001
A2	UNINCUBATED	.002	.002	.001	.001
B1	UNINCUBATED	.002	.011	.001	0
B2	GA	.002	.104	0	0
C1	UNINCUBATED	.006	.001	0	0
C2	ABA	0	.002	0	0
D1	UNINCUBATED	.001	.002	0	0
D2	GA+ABA	.001	.001	0	.001
E1	UNINCUBATED	.002	.001	0	.001
E2	BUFFER	.004	.002	0	0
F1	GA	.002	.001	.012	.001
F2	GA	.004	.002	0	0
G1	GA	.002	.001	.062	0
G2	ABA	.003	.002	0	.001
H1	GA	.003	.001	.057	.001
H2	GA+ABA	.001	.001	0	.050
I1	GA	.001	0	0	.001
I2	BUFFER	.001	0	0	.002
J1	ABA	.002	.001	0	0
J2	ABA	.003	.001	0	0
K1	ABA	.002	0	0	.001
K2	GA+ABA	.001	.001	.001	0
L1	ABA	.001	0	0	.001
L2	BUFFER	.001	0	.001	0
M1	GA+ABA	.001	0	.001	.002
M2	GA+ABA	.002	.001	.001	.003
N1	GA+ABA	.001	0	.001	.002
N2	BUFFER	.002	.001	0	.002
O1	BUFFER	.001	0	0	.002
O2	BUFFER	.001	0	0	.123

MEANS	EAR				
TREATMENT	1	2	3	4	MEAN
UNINCUBATED	.003	.003	.001	0	.002
BUFFER	.002	.001	0	.021	.006
GA	.002	.018	.022	0	.011
GA+ABA	.001	.001	.001	.010	.003
ABA	.002	.001	0	.001	.001
OVERALL	.002	.005	.005	.007	.005

50 DAA

		EAR			
	TREATMENT	1	2	3	4
A1	UNINCUBATED	.001	.002	.001	.010
A2	UNINCUBATED	.002	.002	0	.010
B1	UNINCUBATED	.002	.002	.001	.004
B2	GA	.002	.002	0	.001
C1	UNINCUBATED	.003	.002	.001	.002
C2	ABA	.002	.002	.001	.001
D1	UNINCUBATED	.002	.002	.001	.005
D2	GA+ABA	.002	.002	0	.002
E1	UNINCUBATED	.002	.002	.001	.007
E2	BUFFER	.002	.001	.001	.001
F1	GA	.002	.001	0	.001
F2	GA	.002	.001	.001	.001
G1	GA	.002	.001	.001	.002
G2	ABA	.002	.001	0	.001
H1	GA	.002	.001	.001	.001
H2	GA+ABA	.002	.002	.001	.003
I1	GA	.001	.001	.006	.003
I2	BUFFER	.002	.001	.001	.001
J1	ABA	.002	.001	.001	.001
J2	ABA	.001	0	.001	.001
K1	ABA	.002	.001	.001	.001
K2	GA+ABA	.002	0	.002	.001
L1	ABA	.002	.001	.001	.002
L2	BUFFER	.001	.002	.001	.002
M1	GA+ABA	.001	.001	.001	.001
M2	GA+ABA	.001	.001	.001	0
N1	GA+ABA	.001	0	.001	.001
N2	BUFFER	.001	.001	.001	.039
O1	BUFFER	.002	.001	.002	.001
O2	BUFFER	.002	.001	.002	.003

MEANS	EAR				
TREATMENT	1	2	3	4	MEAN
UNINCUBATED	.002	.002	.001	.006	.003
BUFFER	.002	.001	.001	.008	.003
GA	.002	.001	.002	.001	.001
GA+ABA	.001	.001	.001	.001	.001
ABA	.002	.001	.001	.001	.001
OVERALL	.002	.001	.001	.004	.002

52 DAA

		EAR			
	TREATMENT	1	2	3	4
A1	UNINCUBATED	.095	.004	.013	.002
A2	UNINCUBATED	.081	.004	.002	.001
B1	UNINCUBATED	0	.001	.001	.002
B2	GA	.006	.002	.001	.001
C1	UNINCUBATED	.001	.003	.001	.003
C2	ABA	.002	.001	.004	.002
D1	UNINCUBATED	.001	.002	.001	.001
D2	GA+ABA	.036	.002	.002	0
E1	UNINCUBATED	.000	.001	.003	.001
E2	BUFFER	.003	.002	.001	.003
F1	GA	.079	.001	0	.003
F2	GA	.072	.002	.002	.004
G1	GA	.139	.013	.001	.001
G2	ABA	.001	.002	.002	.002
H1	GA	.166	.002	.068	.005
H2	GA+ABA	.102	.001	.001	0
I1	GA	.225	.001	.004	.006
I2	BUFFER	.004	0	.002	.003
J1	ABA	.002	.001	0	.001
J2	ABA	.002	0	0	.002
K1	ABA	.002	.001	.002	.002
K2	GA+ABA	.135	.001	.001	0
L1	ABA	.009	0	.003	.002
L2	BUFFER	.052	.001	.005	.002
M1	GA+ABA	.058	.001	.003	.001
M2	GA+ABA	.018	0	.002	0
N1	GA+ABA	.001	.001	.030	.003
N2	BUFFER	.002	.001	0	.001
O1	BUFFER	.001	.001	.056	.000
O2	BUFFER	.002	.003	.009	.001

MEANS	EAR				
TREATMENT	1	2	3	4	MEAN
UNINCUBATED	.030	.003	.004	.002	.009
BUFFER	.011	.001	.012	.002	.006
GA	.114	.004	.013	.003	.034
GA+ABA	.058	.001	.006	.001	.017
ABA	.003	.001	.002	.002	.002
OVERALL	.043	.002	.007	.002	.014

53 DAA

	TREATMENT	EAR 1	2	3	4
A1	UNINCUBATED	.003	.002	.002	.002
A2	UNINCUBATED	.002	.003	.002	.001
B1	UNINCUBATED	.001	.001	.002	.003
B2	GA	.019	.005	.001	.009
C1	UNINCUBATED	.003	.001	.010	.003
C2	ABA	.001	.002	.002	.005
D1	UNINCUBATED	.001	.005	.004	.003
D2	GA+ABA	.005	.003	.001	.006
E1	UNINCUBATED	.033	.003	.010	.002
E2	BUFFER	.002	.003	.002	.004
F1	GA	.524	.001	.001	.029
F2	GA	.115	.002	0	.007
G1	GA	.386	.009	.001	.416
G2	ABA	.022	.001	.002	.259
H1	GA	.400	.019	.001	.068
H2	GA+ABA	.015	.003	.001	.004
I1	GA	.111	.003	.001	.900
I2	BUFFER	.002	.002	.002	.006
J1	ABA	.001	.001	.002	.006
J2	ABA	.089	.002	.002	0
K1	ABA	.001	.001	.002	.118
K2	GA+ABA	.008	.001	.002	.815
L1	ABA	.006	.001	.002	.002
L2	BUFFER	.005	0	.003	.002
M1	GA+ABA	.002	.001	.006	.002
M2	GA+ABA	.002	.002	.001	.002
N1	GA+ABA	.002	.001	.001	.001
N2	BUFFER	.002	.002	.002	.002
O1	BUFFER	.003	.001	.001	.041
O2	BUFFER	.003	.002	.002	.005

MEANS	EAR 1	2	3	4	MEAN
TREATMENT					
UNINCUBATED	.007	.002	.005	.002	.004
BUFFER	.003	.002	.002	.010	.004
GA	.259	.006	.001	.238	.126
GA+ABA	.006	.002	.002	.138	.037
ABA	.020	.001	.002	.065	.022
OVERALL	.059	.003	.002	.091	.039

54 DAA

		EAR			
	TREATMENT	1	2	3	4
A1	UNINCUBATED	.002	0	.008	.002
A2	UNINCUBATED	.002	.001	.007	.002
B1	UNINCUBATED	.007	.002	.001	.002
B2	GA	.003	.001	0	.002
C1	UNINCUBATED	.006	.001	.005	.002
C2	ABA	.002	.001	.001	.001
D1	UNINCUBATED	.009	.001	.012	.001
D2	GA+ABA	.002	.001	.003	.001
E1	UNINCUBATED	.006	.001	.002	.001
E2	BUFFER	.003	0	.005	.001
F1	GA	.002	.117	0	.001
F2	GA	.002	.040	.001	.001
G1	GA	.004	.088	.001	.003
G2	ABA	.003	.002	.001	.001
H1	GA	.094	.132	.148	.002
H2	GA+ABA	.012	.662	.001	.002
I1	GA	.003	.036	.001	.002
I2	BUFFER	.002	.001	.001	.001
J1	ABA	.003	0	.001	.001
J2	ABA	.003	.137	.003	.002
K1	ABA	.003	0	0	.001
K2	GA+ABA	.007	.002	0	.001
L1	ABA	0	.165	0	.001
L2	BUFFER	.001	.082	.001	.001
M1	GA+ABA	.001	.001	.001	.002
M2	GA+ABA	0	.001	.004	.001
N1	GA+ABA	.002	.008	.001	.001
N2	BUFFER	.001	.062	.001	.001
O1	BUFFER	.002	0	.002	.002
O2	BUFFER	.001	.001	.003	.001

MEANS	EAR				
TREATMENT	1	2	3	4	MEAN
UNINCUBATED	.005	.001	.006	.002	.003
BUFFER	.002	.024	.002	.001	.007
GA	.018	.069	.025	.002	.028
GA+ABA	.004	.113	.002	.001	.030
ABA	.002	.051	.001	.001	.014
OVERALL	.006	.052	.007	.001	.017

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		EAR			
	TREATMENT	1	2	3	4
A1	UNINCUBATED	.003	.115	.001	.010
A2	UNINCUBATED	.001	.003	.002	.005
B1	UNINCUBATED	.007	.002	.002	.002
B2	GA	.001	.001	.001	.002
C1	UNINCUBATED	.009	.013	.002	.002
C2	ABA	.002	.003	.001	.009
D1	UNINCUBATED	0	.002	.003	.020
D2	GA+ABA	.002	.005	.001	.033
E1	UNINCUBATED	.002	.004	.003	.010
E2	BUFFER	0	.002	.002	.013
F1	GA	0	.507	.002	.006
F2	GA	0	.169	.002	.002
G1	GA	.002	.095	.001	.003
G2	ABA	.002	.002	.003	.001
H1	GA	.002	.004	.002	.013
H2	GA+ABA	.001	.003	.000	.002
I1	GA	.001	.002	.001	.001
I2	BUFFER	.057	.001	0	.001
J1	ABA	.003	.001	.001	.002
J2	ABA	.003	.001	.001	.011
K1	ABA	.001	.003	.001	.005
K2	GA+ABA	.009	.005	.002	0
L1	ABA	0	.001	.002	.001
L2	BUFFER	.453	.004	.002	0
M1	GA+ABA	.002	0	.001	.001
M2	GA+ABA	.002	.001	.002	.002
N1	GA+ABA	.004	.005	.001	.005
N2	BUFFER	.005	.013	0	0
O1	BUFFER	.001	.001	.002	.014
O2	BUFFER	.002	.002	.001	.006

MEANS	EAR				
TREATMENT	1	2	3	4	MEAN
UNINCUBATED	.004	.023	.002	.008	.009
BUFFER	.104	.004	.001	.006	.029
GA	.001	.130	.001	.005	.034
GA+ABA	.003	.003	.001	.007	.004
ABA	.002	.002	.002	.005	.003
OVERALL	.020	.032	.002	.006	.015

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		EAR			
	TREATMENT	1	2	3	4
A1	UNINCUBATED	.001	.012	.016	1.393
A2	UNINCUBATED	0	.003	.008	.006
B1	UNINCUBATED	0	.001	.002	1.501
B2	GA	.001	.062	.002	.156
C1	UNINCUBATED	0	.012	.004	.133
C2	ABA	0	.003	.002	.002
D1	UNINCUBATED	.001	.002	.001	.002
D2	GA+ABA	0	.002	.002	.001
E1	UNINCUBATED	0	.003	.002	.001
E2	BUFFER	.002	.004	.002	.039
F1	GA	0	.001	.003	1.825
F2	GA	.053	.011	.003	.042
G1	GA	.001	.002	.025	.157
G2	ABA	.001	.001	.010	.002
H1	GA	.001	.001	.003	.662
H2	GA+ABA	.001	.001	.002	.016
I1	GA	.006	.007	.002	1.734
I2	BUFFER	0	.001	.001	.084
J1	ABA	0	.005	.001	.004
J2	ABA	.001	.002	.001	.001
K1	ABA	.006	.001	.002	.002
K2	GA+ABA	.002	.001	.002	.010
L1	ABA	.002	0	.002	.010
L2	BUFFER	.002	0	.001	.026
M1	GA+ABA	.002	.001	.002	.001
M2	GA+ABA	.001	.001	.003	.001
N1	GA+ABA	.007	.001	.003	.151
N2	BUFFER	.005	0	.002	.019
O1	BUFFER	.002	0	.001	.052
O2	BUFFER	.001	.002	.002	0

MEANS	EAR				
TREATMENT	1	2	3	4	MEAN
UNINCUBATED	0	.006	.006	.506	.129
BUFFER	.002	.001	.001	.037	.010
GA	.010	.014	.006	.763	.198
GA+ABA	.002	.001	.002	.030	.009
ABA	.002	.002	.003	.003	.002
OVERALL	.003	.005	.004	.268	.070

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		EAR			
	TREATMENT	1	2	3	4
A1	UNINCUBATED	.005	0	.001	.020
A2	UNINCUBATED	.002	.001	.003	.005
B1	UNINCUBATED	.002	.003	.002	.002
B2	GA	.020	.052	.002	.001
C1	UNINCUBATED	.001	0	.002	.001
C2	ABA	.001	.001	.001	.001
D1	UNINCUBATED	.001	.002	.001	.001
D2	GA+ABA	.002	.001	.002	.001
E1	UNINCUBATED	.023	0	0	.008
E2	BUFFER	.003	.001	.002	.007
F1	GA	.001	.002	0	.018
F2	GA	.003	.001	.005	.001
G1	GA	.002	.001	.603	.002
G2	ABA	.002	.001	0	.001
H1	GA	1.175	.149	.001	.001
H2	GA+ABA	.003	.002	.003	.001
I1	GA	.002	0	.002	.003
I2	BUFFER	.001	.004	.006	.001
J1	ABA	.001	.009	.001	.001
J2	ABA	.001	.002	.001	.001
K1	ABA	0	.001	.002	.001
K2	GA+ABA	.001	.002	.001	.001
L1	ABA	.001	0	.002	.002
L2	BUFFER	0	.001	.001	.001
M1	GA+ABA	.001	.005	.001	.001
M2	GA+ABA	.001	.002	.003	.001
N1	GA+ABA	.002	.011	.002	.001
N2	BUFFER	.001	.002	.001	.001
O1	BUFFER		.003	.002	.002
O2	BUFFER	.001	.001	.001	.002

MEANS		EAR			
TREATMENT	1	2	3	4	MEAN
UNINCUBATED	.006	.001	.001	.006	.004
BUFFER	.001	.002	.002	.002	.002
GA	.200	.034	.102	.004	.085
GA+ABA	.001	.004	.002	.001	.002
ABA	.001	.002	.001	.001	.001
OVERALL	.042	.009	.023	.003	.019